Pax Genes in *Drosophila* Myogenesis: Their Functions and Regulation

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Summary

To decipher a gene network controlling a developmental process, it is important to understand both the functions of the genes crucial for this developmental process and the molecular mechanisms regulating their expression. In this thesis, I studied the mechanisms regulating cell fate determination during *Drosophila* embryonic myogenesis by analyzing the functions of the Pax genes *Pox meso* (*Poxm*) and *gooseberry* (*gsb*) in muscle formation and characterizing the mechanisms through which *Poxm* is repressed by the TGFβ superfamily member Dpp.

Although in vertebrates, *Pax1/9* and *Pax3/7* are required for osteogenesis and myogenesis respectively, their homologs *Poxm* and *gsb* are both involved in *Drosophila* embryonic myogenesis. During early embryonic stages, Poxm is expressed in a lateral-ventral domain of the somatic mesoderm, and cells expressing mesodermal Gsb are within this early-Poxm-expressing domain. During late embryonic stages, while the expression of Poxm is retained in differentiated muscle fibers, the expression of Gsb can no longer be detected. In both *Poxm* and *gsb* loss-of-function mutants, ventral and lateral muscles are affected most strongly, with the muscle phenotypes of *gsb* mutants being more severe. However, when overexpressed throughout the mesoderm, Poxm results in a severely altered muscle pattern while Gsb only affects a few lateral muscles. The muscle phenotypes observed in *Poxm* null mutants are largely caused by the absence of *Poxm* during early myogenic stages, and this early function of *Poxm* is partially redundant with the functions of *l(1)sc* in myogenesis. During late myogenic stages, *Poxm* functions as a muscle identity gene to regulate muscle founder specification and muscle differentiation. While the activation of *Poxm* requires *gsb*, the expression of Gsb in the mesoderm is independent of both Poxm and the Six family homeodomain protein D-Six4. Taken together, these results indicate that both *Poxm* and *gsb* are key players performing partially overlapping functions in *Drosophila* embryonic myogenesis, and they are part of the gene network regulating cell fate specification in the somatic mesoderm during early embryonic stages.

The expression of Poxm is restricted to ventral-lateral mesoderm by Dpp signaling, which represses it dorsally. While independent of the co-repressor Shn, this repression is mediated by the Dpp receptor TKV and the R-SMAD MAD through the activation of the Dpp signaling pathway in mesoderm cells. *Poxm* is also repressed by Dpp that is ectopically expressed ventrally. In both cases, a 280 bp *cis*-regulatory element of *Poxm*, *um2I&2II*, responds to the activated Dpp signaling that leads to gene repression. These results raise the possibility that molecular mechanisms independent of Shn may act on the enhancers of certain Dpp target genes to repress transcription in response to Dpp signaling.

Zusammenfassung

Um ein Gennetzwerk, das einen Entwicklungsprozess steuert, aufzuklären, ist es wichtig, sowohl die Funktionen der daran entscheidend beteiligten Gene wie auch die molekularen Mechanismen, die ihre Expression regulieren, zu verstehen. In dieser Dissertation habe ich die Mechanismen untersucht, die die zellulären Schicksale während der Myogenese im *Drosophila* Embryo kontrollieren, indem ich die Funktionen der Pax Gene *Pox meso* (*Poxm*) and *gooseberry* (*gsb*) während der Muskelbildung analysiert und die Mechanismen charakterisiert habe, durch die *Poxm* von Dpp, das der TGFβ Superfamilie angehört, reprimiert wird.

Obschon in Wirbeltieren *Pax1/9* und *Pax3/7* in der Knochen- und Muskelbildung notwendig sind, kommen in *Drosophila* beide homologen Gene, *Poxm* und *gsb*, in der Muskelbildung während der Embryogenese zur Anwendung. In den frühen embryonalen Stadien wird Poxm in einer lateralen-ventralen Domäne des somatischen Mesoderms exprimiert, während die Zellen, die Gsb im Mesoderm exprimieren, innerhalb dieser Domäne liegen. In den späten embryonalen Stadien wird Poxm in bestimmten differenzierten Muskelfasern exprimiert, während Gsb nicht mehr exprimiert wird. Sowohl in *Poxm* und *gsb* 'loss-of-function' Mutanten sind die ventralen und lateralen Muskeln am stärksten betroffen, wobei die Phänotypen der *gsb* Mutanten schwerwiegender sind. Wird jedoch Poxm oder Gsb im gesamten Mesoderm überexprimiert, erzeugt Poxm ein deutlich verändertes Muskelmuster, während Gsb nur wenige laterale Muskeln verändert. Die Muskelphänotypen, die man in *Poxm* Nullmutanten beobachtet, werden hauptsächlich durch das Fehlen von *Poxm* während den frühen embryonalen Stadien verursacht, und diese frühe Funktion von *Poxm* ist mit den Funktionen von *l(1)sc* während der Myogenese teilweise redundant. Während den späten Stadien der Myogenese agiert *Poxm* als ein Muskelidentitätsgen, um die Spezifizierung und Differenzierung der betroffenen Muskeln zu regulieren. Während die Aktivierung des *Poxm* Gens von *gsb* abhängt, ist die Expression von Gsb im Mesoderm sowohl von Poxm wie auch von dem der Six Familie angehörigen Homöodomänen-Protein D-Six4 unabhängig.

Insgesamt zeigen diese Resultate, dass sowohl *Poxm* als auch *gsb* während der embyronalen Myogenese in *Drosophila* Schlüsselrollen spielen und teilweise überlappende Funktionen ausüben und dass sie Teil eines Gennetzwerkes sind, das die Schicksale der Zellen im somatischen Mesoderm während den frühen embryonalen Stadien reguliert.

Die Expression von *Poxm* wird durch das Dpp Signal, das *Poxm* dorsal reprimiert, auf das ventrale-laterale Mesoderm beschränkt. Diese Repression ist unabhängig vom Korepressor Shn, verläuft aber dennoch über die Aktivierung des Dpp Signaltransduktionswegs via den Dpp-Rezeptor TKV und den R-SMAD Effektor MAD in den mesodermalen Zellen. *Poxm* wird ebenfalls von Dpp reprimiert, wenn dieses ventral ektopisch exprimiert wird. In beiden Fällen reagiert ein 280 Bp *cis*-regulatorisches Element von *Poxm*, *um2I&2II*, auf die aktivierte Dpp Signaltransduktion, die zur Repression von *Poxm* führt. Aufgrund dieser Resultate muss man die Möglichkeit in Betracht ziehen, dass von Shn unabhängige molekulare Mechanismen die Enhancer gewisser Dpp-Zielgene beeinflussen, um deren Transkription in Abhängigkeit des Dpp Signals zu reprimieren.

Chapter 1

General Introduction

A key question in developmental biology is how specific cell fates are established by temporal and spacial cues through precise genetic regulations. The specification of the mesoderm in *Drosophila melanogaster* provides an excellent paradigm to study how cells that are initially identical can acquire different fates by expressing specific sets of cell-type regulators. Several organs can arise from the *Drosophila* mesoderm, including the muscles of the gut (visceral muscles), the body wall muscles (somatic muscles), the heart, the fat body, and the gonads (Bate, 1993). Thus, the selection of cells undergoing myogenesis is part of a more general process in which the progenitors of different mesodermal tissues are formed. Furthermore, since the *Drosophila* larva has a remarkably complex muscle pattern, which is formed when individual muscles are seeded and specified from cells of the myogenic lineage (Bate, 1993), studies on how different muscles get their unique identities may also provide important information to the understanding of cell fate determination in general.

On the whole, it is clear that the fate of a specific muscle is determined, at least partially, by the expression of a relatively small set of cell fate regulators, the so-called muscle identity genes, which are activated by a combination of signaling and transcription factors in response to positional information (reviewed by Bate, 1993; Baylies et al., 1998). Although many of these muscle identity genes have been identified, the mechanisms that activate their transcription are still poorly understood due to a lack of knowledge on the identity and functions of the upstream regulators.

Pax genes encode transcription factors containing a characteristic 128-amino acid paired domain that confers sequence-specific DNA binding. The first Pax genes were isolated from *Drosophila* (Bopp et al., 1986), and this gene family is conserved in many animal species (reviewed by Noll, 1993). Through years of study, Pax genes have been shown to play key roles in tissue formation and organ development

(reviewed by Buckingham and Relaix, 2007). Among them, *Pax3* and *Pax7* in particular, are crucial for myogenesis in vertebrates (reviewed by Tajbakhsh and Buckingham, 2000). In addition, *Pax1* and *Pax9*, whose expression patterns partially overlap with those of *Pax3* and *Pax7*, play important roles in the formation of cartilage and bone, which like the muscles are derived from the somites (Borycki & Emerson, 1997; Peters et al., 1999).

Like vertebrates, *Drosophila* divides its mesoderm into segmental units, which are similar to vertebrate somites. Interestingly, *Pox meso* (*Poxm*), a member of the *Pax1/9* subfamily and *gooseberry* (*gsb*), a member of the *Pax3/7* subfamily are expressed in these segmental mesoderm units. Considering the functions of their homologs in vertebrate, it would be interesting to study the functions of *Poxm* and *gsb* in *Drosophila* myogenesis. Moreover, being able to put *Poxm* and *gsb* in appropriate positions in the regulatory network of *Drosophila* myogenesis will also shed light on analyzing the functions of their homologs in vertebrate development.

Although *Pox meso* (*Poxm*) was the first and so far only gene whose initial expression was shown to occur specifically in the anlage of the somatic mesoderm, its role in somatic myogenesis remained unknown. In chapter 2, I tried to analyze the mesodermal expression pattern of *Poxm* in detail and dissect its functions associated with these distinct expression patterns.

In chapter 3, I discussed the functions of *gsb* in *Drosophila* embryonic myogenensis and revealed the regulatory and functional relationships between *gsb* and *Poxm* in the broad context of the myogenic regulatory networks.

Not only is our knowledge of the upstream regulators themselves quite limited, but also relatively little is known about how they are regulated by different signalings and positional information. An example is the repression of *Poxm* by Dpp, a TGFβ/BMP superfamily member in *Drosophila*, which was reported by Staehling-Hampton et al. nearly 14 years ago (Staehling-Hampton et al., 1994). However, how this repression is performed still remains a mystery. In chapter 4, I tried to answer this question by isolating specific enhancers responsive to Dpp signaling in the upstream regulatory region of *Poxm* and analyzing the genetic interactions between *Poxm* and components of the Dpp signaling pathway.

References

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster*, Vol. 2, Bate, M. and Martinez-Arias, A., eds. (Cold Spring Harbor, NY: CSH Laboratory Press), pp. 1013-1090.

Baylies, M.K., Bate, M. and Gomez, M.R. (1998). Myogenesis: A view from *Drosophila*. *Cell* 93, 921-927.

Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. and Noll, M. (1986). Conservation of a large protein domain in the segmentation gene *paired* and in functionally related genes of *Drosophila*. *Cell* 47, 1033-1040.

Borycki, A.G. and Emerson, C.P. (1997). Muscle determination: another key player in myogenesis? *Curr. Biol.* 7, R620–623.

Buckingham, M. and Relaix, F. (2007). The role of *Pax* genes in the development of tissues and organs: *Pax3* and *Pax7* regulate muscle progenitor cell functions. *Annu. Rev. Cell Dev. Biol.* 23, 645-673.

Noll, M. (1993). Evolution and role of *Pax* genes. *Curr. Opin. Genet. Dev.* 3, 595-605.

Peters, H., Wilm, B., Sakai, N., Imai, K., Maas, R. and Balling, R. (1999). Pax1 and Pax9 synergistically regulate vertebral column development. *Development* 126, 5399–5408.

Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E. and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* 372, 783-786.

Tajbakhsh, S. and Buckingham, M. (2000). The birth of muscle progenitor cells in the mouse: spatiotemporal considerations. *Curr. Top. Dev. Biol.* 48, 225–268.

Chapter 2

A key role of *Pox meso* in somatic myogenesis of *Drosophila*. *Development* 134, 3985-3997 (2007).

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A key role of *Pox meso* in somatic myogenesis of *Drosophila*

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The Pax gene *Pox meso* (*Poxm*) was the first and so far only gene whose initial expression was shown to occur specifically in the anlage of the somatic mesoderm, yet its role in somatic myogenesis remained unknown. Here we show that it is one of the crucial genes regulating the development of the larval body wall muscles in *Drosophila*. It has two distinct functions expressed during different phases of myogenesis. The early function, partially redundant with the function of *lethal of scute* [*l(1)sc*], demarcates the 'Poxm competence domain', a domain of competence for ventral and lateral muscle development and for the determination of at least some adult muscle precursor cells. The late function is a muscle identity function, required for the specification of muscles DT1, VA1, VA2 and VA3. Our results led us to reinterpret the roles of *l(1)sc* and *twist* in myogenesis and to propose a solution of the '*l(1)sc* conundrum'.

KEY WORDS: *Drosophila*, *Pox meso*, Pax gene, *lethal of scute* conundrum, Somatic myogenesis, Muscle progenitors, Muscle patterning

INTRODUCTION

The development of the complex pattern of the larval body wall muscles of *Drosophila* provides an excellent paradigm of how a final pattern is established through precise genetic control (reviewed by Bate, 1993; Baylies et al., 1998). Each of the abdominal hemisegments A2-A7 has 30 identifiable individual muscles (Bate, 1993) that develop from the somatic mesoderm. This process is initiated when the invaginated mesoderm migrates dorsolaterally under the ectoderm (Beiman et al., 1996; Gisselbrecht et al., 1996) and is prepatterned by the segmentation genes (Lee and Frasch, 2000; Riechmann et al., 1997): the product of *sloppy paired* (*slp*), whose activity is maintained by the ectodermal Wingless (Wg) signal, restricts high levels of the bHLH transcription factor Twist (Twi) to the mesodermal regions below the posterior portions of the ectodermal parasegments (Baylies et al., 1998). These high levels of Twi function as a myogenic switch, separating the posterior somatic and cardiac mesoderm from the anterior visceral mesoderm and fat body (Baylies and Bate, 1996; Dunin Borkowski et al., 1995). When the dorsal migration of the mesoderm is complete, these metamerically repeated Slp or high Twi domains are further subdivided along the dorsoventral axis by the ectodermal signal Dpp (Staehling-Hampton et al., 1994). This signal restricts transcription of *tinman* (*tin*) to the dorsal mesoderm, where its homeodomain protein specifies heart and dorsal somatic mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990; Frasch, 1995). However, the determinant of the non-dorsal somatic mesoderm remains largely unknown. It appears that Pox meso (Poxm) expression is restricted to the ventral part of the high Twi domain by Dpp (Staehling-Hampton et al., 1994) to define the lateral and ventral somatic mesoderm anlage. The characterization of the role of *Poxm* in somatic myogenesis is therefore expected to fill an important gap in our understanding of the gene network regulating this process.

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Soon after this subdivision of the mesoderm, the proneural gene *lethal of scute* [*l(1)sc*] begins to be expressed in at least 19 promuscular clusters of cells within the high Twi domain (Carmena et al., 1995). From these clusters, muscle progenitors are singled out by lateral inhibition through Notch (N) and Ras signaling and are specified by the expression of muscle-identity genes (Buff et al., 1998; Carmena et al., 1995; Carmena et al., 1998a; Carmena et al., 2002; Michelson et al., 1998; Stathopoulos et al., 2004). Cells not singled out begin to express the zinc finger protein Lame duck (Lmd; also known as Minc), which specifies them as fusion-competent myoblasts (FCMs) (Duan et al., 2001; Ruiz-Gómez et al., 2002). The progenitors divide to generate different muscle founders, a muscle founder and an adult muscle precursor, or a founder and a cell producing either two adult muscle precursors or two pericardial cells (Carmena et al., 1995; Carmena et al., 1998b; Jagla et al., l998; Nose et al., 1998; Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997). Each founder forms an individual syncytial muscle precursor by fusing with neighboring FCMs. One of the key steps in muscle pattern formation is the specification of a muscle founder by the expression of a specific set of muscle identity genes (Bate, 1990; Bour et al., 2000; Dohrmann et al., 1990; Ruiz-Gómez et al., 2000; Rushton et al., 1995). Although an increasing number of these genes have been identified in recent years, the mechanisms that activate their transcription are still poorly understood. Hence, it is important to identify the genes whose products directly regulate the muscle identity genes.

In this study, we describe the functional characterization of the *Poxm* gene. *Poxm* belongs to the Pax gene family whose members encode transcription factors, including a paired domain (Bopp et al., 1989) (reviewed by Noll, 1993). The temporal and spatial expression patterns of *Poxm* and its loss- and gain-of-function phenotypes reported here demonstrate that it is required for most ventral and lateral abdominal muscles to develop properly in all segments and for the activation of muscle identity genes. In addition, *Poxm* acts itself as muscle identity gene in a few muscles and thus plays a dual role in somatic myogenesis.

MATERIALS AND METHODS

Generation of transgenic flies

To generate transgenic *Poxm-Gal4* lines, an 8.4 kb *Eco*RI fragment (most distal *Eco*RI fragment of P106, see Fig. S1A in the supplementary material) or a 1.8 kb *Xba*I-*Bam*HI fragment (from P111, see Fig. S1A in

the supplementary material; *Bam*HI site 93 bp upstream of third upstream *Eco*RI site in Fig. 3E) of the *Poxm* upstream region was cloned into a pBlueScript pKS$^+$ vector with an altered polylinker that included two *Not*I sites. These fragments were removed by *Not*I digestion and inserted into the *Not*I site of the P-element vector pDA188 (kindly provided by Konrad Basler; K. Basler, unpublished) to produce the *Poxm8.4-Gal4* and *Poxm1.8-Gal4* constructs used to generate the corresponding transgenic lines. To generate *Poxm-lacZ* lines, the 1.8 kb and 8.4 kb fragments mentioned above were removed by *Not*I digestion and inserted together with the *Poxm* promoter/leader region (–333 to +700, a *Not*I-*Kpn*I fragment, generated by PCR from a genomic DNA clone) between the *Not*I and *Kpn*I cloning sites of the pWZ.1 P-element vector (Gutjahr et al., 1994). To produce transgenic *UAS-Poxm* lines, the 2.5 kb full-length *Poxm* cDNA, P29c1, was inserted into the *Eco*RI site of the pUAST vector (Brand and Perrimon, 1993).

To generate transgenic *um1-2-Poxm* lines, an *Xba*I-*Xho*I genomic fragment, extending from the upstream *Xba*I site to the 5′ leader (Fig. 3E), and the adjacent 2.5 kb *Xho*I-*Pst*I *Poxm*-cDNA fragment of P29c2, extending from the leader to the 3′ trailer beyond the first poly(A) addition site (see Fig. S1A in the supplementary material), were cloned between the *Xba*I and *Pst*I sites of the P-element vector PW6 (Klemenz et al., 1987).

For germline transformation, these constructs, all verified by DNA sequencing, were coinjected with the transposase carrying plasmid P(Δ2-3) into w^{1118} or *y w* embryos. Three to five independent lines of each construct were established and analyzed.

Immunohistochemistry and microscopy

To produce an anti-Poxm antiserum, a *Poxm*-cDNA fragment encoding the 234 amino acids C-terminal to the paired-domain was cloned between the *Bam*HI and *Eco*RI sites of the pGEX-3X GST-fusion vector (Pharmacia). The fusion protein was produced in bacteria, purified, and used for immunization of rabbits as described previously (Gutjahr et al., 1993a). Antiserum was collected, affinity-purified, and used at a 1:10 dilution for histochemical detection of Poxm as described (Gutjahr et al., 1993a). The purified anti-Poxm antiserum is free of any crossreactivity with embryonic antigens as verified in homozygous $Poxm^{R361}$ embryos.

The following primary antisera were also used: rabbit anti-MHC [myosin heavy chain (Kiehart and Feghali, 1986)], rat anti-Slou (Carmena et al., 1995), rabbit anti-Twi (Roth et al., 1989), rat anti-L(1)sc (provided by Ana Carmena, Instituto de Neurociencias, Alicante, Spain), rabbit anti-β-galactosidase (Cappel), rabbit anti-Tin (Yin and Frasch, 1998), and rabbit anti-GFP (Medical & Biological Laboratories, Nagoya, Japan). Embryos were fixed and stained as described previously (Gutjahr et al., 1993a).

Muscle patterns were visualized after staining with anti-MHC (or with anti-β-galactosidase, when expressed under indirect control of *Poxm*) under bright-field microscopy by a Zeiss Axiophot. The fluorescent signals of double-labeled embryos were amplified by tyramide signal amplification (TSA; kits #12 and #25 from Invitrogen), and embryos were analyzed by a Leica SP1 confocal microscope.

Fly stocks

The following fly stocks were used. Oregon-R (Munich). *Df(3R)dsx*$^{D+R5}$*/TM3, Sb* (Baker and Wolfner, 1988). *Df(3R)dsx*$^{M+R29}$*/TM3, Sb* (Deák et al., 1997). *UAS-lacZ* (Bloomington stock 1777). *24BGal4* (Bloomington stock 1767). *UAS-GFPnls* (Bloomington stock 4775). *w*; Df(3R)159/TM3, Sb P{ry*$^+$*; hb-lacZ}*. w^{1118}; $Poxm^{R361}$ *red/TM3, Sb Ser P{w*$^+$*; hb-lacZ}*. *y w*; *Poxm8.4-Gal4/TM6B*. *y w*; *Poxm1.8-Gal4* (2nd chromosome). *y w*; *um1-2-Poxm*; $Poxm^{R361}$ *red/TM3, Sb P{ry*$^+$*; hb-lacZ}. UAS-Poxm* (3rd chromosome). *Df(1)sc*^{19}*/FM7, P{ry*$^+$*; ftz-lacZ}. Df(1)sc*^{19}*/FM7, P{ry*$^+$*; ftz-lacZ}*; $Poxm^{R361}$ *red/TM3, Sb Ser P{w*$^+$*; hb-lacZ}. w*; l(3)S028206b*^{S028206b19}*/TM3, Sb* (Deák et al., 1997). *w*; P{Mhc-tauGFP}/TM6B* (Chen et al., 2003). *P{PZ}rP298*; *ry*^{506} (Nose et al., 1998). *w*; *lmd*^{1}*/TM6B* (Duan et al., 2001). *Dmef2*$^{22\text{-}21}$*/CyO* (Bour et al., 1995). *y w*; *Poxm1.8-lacZ* (3rd chromosome). *y w*; *Poxm8.4-lacZ* (3rd chromosome).

RESULTS

Structure of the *Poxm* gene and its predicted protein sequence

The *Poxm* gene has been cloned on the basis of its homology to the paired box of the *paired* (*prd*) and *gooseberry* (*gsb*) genes (Bopp et al., 1986), and was mapped to chromosomal band 84F11-12 (Bopp et al., 1989). It extends over more than 20 kb that include two exons and many cis-regulatory elements located in the upstream region and the large intron (see Fig. S1A in the supplementary material) (Bopp et al., 1989). The Poxm protein, predicted from the longest open reading frame, consists of 370 amino acids and includes a paired domain close to its N terminus and an octapeptide in its C-terminal moiety (see Fig. S1B in the supplementary material) (Bopp et al., 1989; Noll, 1993). Except for its first 10 base pairs, the open reading frame is encoded entirely by exon 2. The paired domain of Poxm belongs to the Pax1/9 class (Bopp et al., 1989; Noll, 1993) and displays 88% identity and 92% similarity to mammalian Pax1/9-type paired domains (Fu and Noll, 1997).

Expression of Poxm in the somatic mesoderm during myogenesis

In agreement with earlier results (Bopp et al., 1989), Poxm protein is localized in the nucleus and first detectable in the somatic mesoderm at early stage 10 (Fig. 1A,B). During stage 10, Poxm becomes expressed in segmentally repeated mesodermal ‘stripes’ underlying the ectodermal parasegments 2-14, in the cephalic mesoderm, the proctodeal anlage and a group of ectodermal cells in the clypeolabrum, which presumably corresponds to part of the esophageal anlage (Fig. 1C). At this stage, the posterior boundaries of mesodermal Poxm coincide with those of ectodermal Gsb (Bopp et al., 1989), which largely coincide with the parasegmental borders (Gutjahr et al., 1993b). Consistent with these calibrations along the anteroposterior axis and those of others (Riechmann et al., 1997), we find that Poxm is expressed in cells of the high Twi domain in the ventral and lateral mesoderm (Fig. 2A-C). Since *Poxm* is repressed in the dorsal portion of each segment by the ectodermal signal Dpp (Staehling-Hampton et al., 1994), the number of Poxm-expressing cells is reduced with decreasing distance from the dorsal margin, thus forming a triangular pattern (Fig. 1D). At this stage, Tin expression is not yet completely restricted to the dorsal mesoderm (Fig. 2D). Whereas high levels of Tin in the dorsal region and Poxm are expressed in complementary patterns, Poxm is coexpressed with low Tin levels in the ventral and lateral regions (Fig. 2D-F). During stage 11 Poxm is restricted to fewer cells, some of which will form subsets of muscle progenitors and cells of the promuscular clusters (Fig. 1E,F), as evident from its partial co-localization with L(1)sc (Fig. 2G-I). During germ band retraction, Poxm disappears from the most anterior mesodermal stripe and the telson (Fig. 1E,G). By stage 12, Poxm expression is maintained only in six cells each of the abdominal segments A1-A7 (Fig. 1H), identified as founders of muscles DO3, DT1 and VA1-VA3, and as ventral adult muscle precursor (VaP) by double-staining of Poxm and Slouch (Slou) (Fig. 2J-L). At this time, it becomes apparent that more cells express Poxm in the ventral regions of the thoracic segments than of the abdominal segments (Fig. 1G). In this study, we focus on the role of *Poxm* in myogenesis of abdominal segments A2-A7.

As myoblast fusion proceeds during stage 13, the number of Poxm-positive nuclei increases (Fig. 1I,J). These coincide with the precursors of muscles DT1 and VA1-3 (Fig. 1J,L-O), identified by double-staining of Poxm and MHC-tauGFP (Myosin heavy chain-tauGFP). During stage 15, Poxm expression begins to be reduced in the ventral clusters and is diminished in the dorsolateral region (Fig.

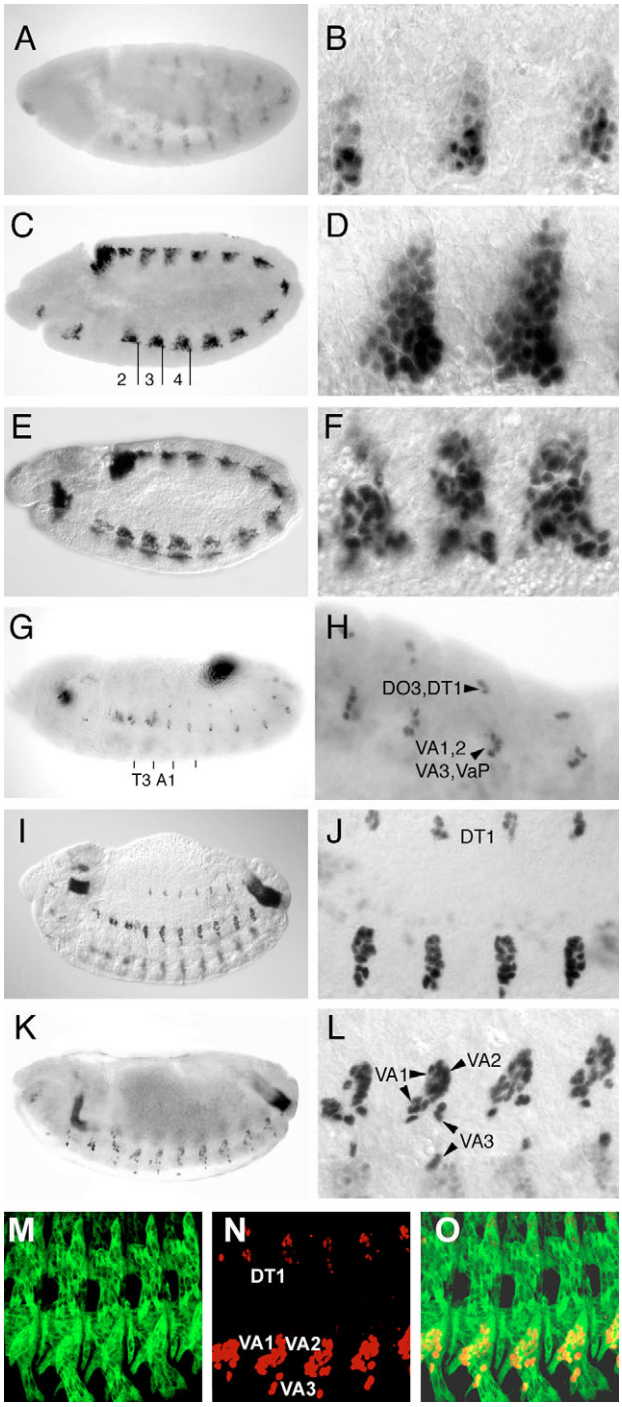


Fig. 1. Expression of Poxm protein in developing larval body wall muscles during embryogenesis. (**A-L**) Whole-mount wild-type embryos were stained with purified anti-Poxm antiserum. Entire embryos (A,C,E,G,I,K) and enlarged views of parasegments 6-8 (B,F,H), 7 and 8 (D), or 6-9 (J,L) at early stage 10 (A,B), late stage 10/early stage 11 (C,D), mid stage 11 (E,F), stage 12 (G,H), stage 13 (I,J) and stage 15 (K,L) are shown. Note that the characteristic triangular pattern of early Poxm expression (D) in parasegments 2-14 is obscured in the overviews (C,E) because not all Poxm-expressing cells are in focus. Therefore, embryos were unfolded and flattened to show enlarged views of parasegments at extended germ band stages (B,D,F) and during germ band retraction (H). Embryos are oriented with their anterior to the left and dorsal side up. At stage 12, Poxm is expressed in the founders of muscles DO3, DT1, VA1-3 and in the ventral adult precursor (VaP; H), and later in the precursors of these muscles except DO3 and VaP (J,L). (**M-O**) Muscles that express Poxm were identified by double-labeling of embryos from *P{Mhc-tauGFP}/TM6B* parents with anti-GFP and anti-Poxm to reveal the muscle pattern (M), the late Poxm expression (N) and the merged image (O). Lateral and ventral muscles in four abdominal hemisegments of a stage 15 embryo with its anterior to the left and dorsal side up are shown. Note that muscles VA1 and VA2 overlap dorsally. For muscle nomenclature, see Bate (Bate, 1993) or Fig. 4J,N.

1K), from which it disappears during stage 16. By stage 17, Poxm is no longer detectable in the mesoderm or any of its derivatives. Outside the mesoderm, particularly striking is its expression in the developing esophagus and hindgut (Fig. 1A,C), where it is maintained at high levels throughout embryogenesis (Fig. 1E,G,I,K).

In summary, Poxm expression in the ventral and lateral portions of the high Twi domain colocalizes with weak Tin expression and is complementary to the high levels of Tin in the dorsal region. Subsequently, its mesodermal expression is confined to fewer cells, some of which will form promuscular clusters and muscle progenitors. Poxm persists in some, but not all of the muscle founders derived from the Poxm-expressing progenitors and is ultimately expressed in four muscle precursors. After the formation of muscle fibers, Poxm disappears. This time course of Poxm expression in developing muscles suggests that *Poxm* functions in somatic myogenesis.

Fate of Poxm-expressing cells during early and late myogenesis

To further analyze the nature and fate of Poxm-expressing cells during early and late myogenesis, *lacZ* was expressed under the indirect control of different *Poxm* upstream regions by the use of the Gal4/UAS system (Brand and Perrimon, 1993). Because of the perdurance of β-galactosidase (β-gal) resulting from (i) the amplification and delay of β-gal inherent in the Gal4/UAS system and (ii) the considerably enhanced stability of both Gal4 and β-gal proteins as compared to that of Poxm, we can follow the fate of cells expressing Poxm during earlier embryonic stages by examining β-gal expression at later stages.

Under the control of a 1.8 kb upstream fragment of *Poxm* (Fig. 3E), β-gal is expressed in a pattern similar, but not identical, to that of early Poxm in the mesoderm (Fig. 3A), presumably because of the temporal delay in expression of the Gal4/UAS system. A similar early expression pattern is observed (Fig. 3C) when *lacZ* is expressed under the control of an 8.4 kb upstream fragment (Fig. 3E). We have also examined β-gal expression under the direct control of the 1.8 kb and 8.4 kb *Poxm* enhancers. In both cases, β-gal and Poxm are coexpressed during early embryonic stages and no ectopic β-gal is detectable (see Fig. S2 in the supplementary material).

Patterns of β-gal expression were then examined at later stages in differentiating muscles. At stage 16, the 8.4 kb fragment supports strong *lacZ* expression in muscles DT1 and VA1-3 (Fig. 3D), in agreement with late Poxm expression, which is restricted to these muscles (Fig. 1N). In addition, however, muscles VL1-4, VO1-6, frequently LT3 and LT4, and occasionally muscle SBM are labeled by β-gal, although at moderate or considerably lower intensities

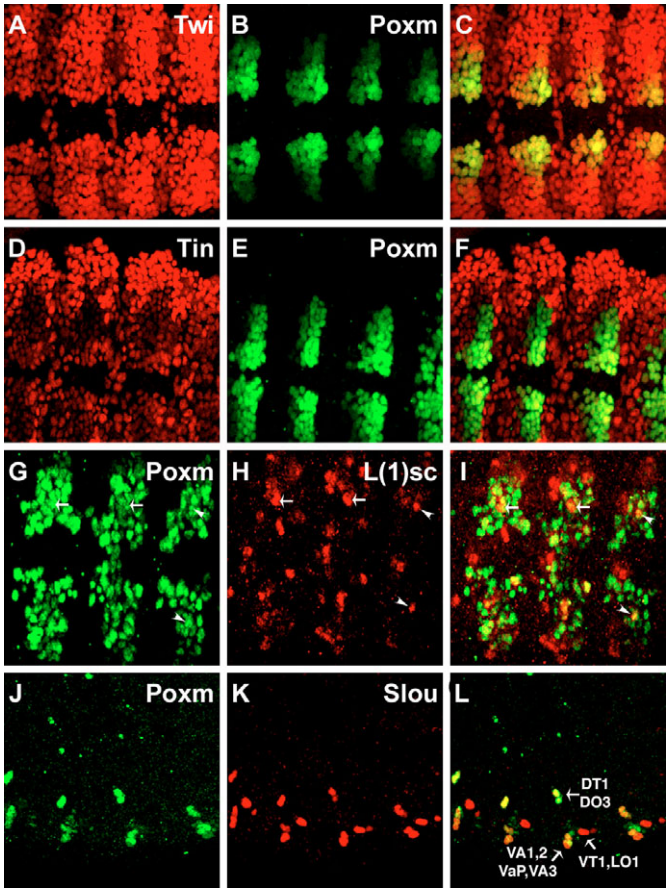


Fig. 2. Early ventral and lateral Poxm expression complementary to dorsal high Tin expression in somatic mesoderm precedes its expression in progenitors of ventral and lateral somatic muscles and in a specific subset of muscle founders. (**A-C**) Early Poxm expression is restricted to the ventral and lateral somatic mesoderm. Poxm expression (B) and high levels of Twi (A), which mark the somatic mesoderm (Dunin Borkowski et al., 1995; Baylies and Bate, 1996) can be seen to coincide in the ventral and lateral regions of the somatic mesoderm (C), as observed by confocal microscopy. A ventral view of four abdominal parasegments of a late stage 10/early stage 11 embryo, oriented with their anterior to the left. (**D-F**) Complementary expression patterns of Poxm and high levels of Tin in the somatic mesoderm. Poxm expression in the ventral and lateral regions (E) abuts high expression levels of Tin in the dorsal region (D), but coincides with lower levels of Tin (F) in an early stage 11 embryo shown in a ventral view. (**G-I**) Poxm is expressed in cells of promuscular clusters and muscle progenitors. Poxm (G) and L(1)sc (H) expression coincide in many ventral and lateral muscle progenitors (I), as observed by double-labeling of Poxm and L(1)sc. Some cell clusters and single progenitors that coexpress L(1)sc and Poxm are indicated by arrows and arrowheads, respectively. Three abdominal parasegments, oriented with their anterior to the left, are shown on both sides of the ventral midline of a mid stage 11 embryo. (**J-L**) Poxm is expressed in specific muscle founders and in the ventral adult precursor. Lateral views of four abdominal hemisegments of a stage 12 embryo double-labeled for late Poxm (J) and Slou (K) expression, oriented with its anterior to the left, revealed by confocal microscopy. In the hemisegment where muscle founders are marked by arrows, expression of late Poxm and Slou coincide (L) in the founders of muscles DO3, DT1, VA2, VA3, and in the ventral adult muscle precursor VaP, whereas Slou has disappeared from the VA1 founder that continues to express Poxm (Carmena et al., 1995; Dohrmann et al., 1990). Expression of Slou in the founders of VT1 and LO1, which do not express Poxm, is also clearly visible (Carmena et al., 1995; Dohrmann et al., 1990).

(Fig. 3D). By contrast, when *lacZ* is expressed under control of the 1.8 kb fragment, it is not detected in muscle DT1 and only at low or moderate levels in muscles VA1-3 (Fig. 3B). It follows that late Poxm expression is under the control of sequences present in the 8.4 kb but not the 1.8 kb fragment (Fig. 3E). Owing to perdurance, when expressed only under control of the early enhancer, β-gal is also observed at moderate or low levels in the ventral muscles VL1-4, VO1-6, frequently in the lateral muscles LT3, LT4, LL1, LO1, SBM and rarely in LT2 and VT1 (Fig. 3B).

These results indicate that cells expressing Poxm early during myogenesis are those from which ventral and lateral muscle progenitors are selected. However, since muscle fibers form by fusion of founders with FCMs, β-gal patterns observed in differentiating muscles may result from the perdurance of β-gal in founders or FCMs. To rule out the possibility that this perdurance is derived exclusively from expression in FCMs, we examined the expression of nuclear GFP under indirect control of the 1.8 kb fragment in lmd^{1} (Duan et al., 2001) or $Dmef2^{22\text{-}21}$ (Bour et al., 1995) mutants, in which fusion is blocked and muscle founders were marked by the *dumbfounded* enhancer trap chromosome rP298-lacZ (Nose et al., 1998). Because of the perdurance of GFP, we can follow the fate of cells expressing early Poxm by examining their expression of GFP at later stages. In lmd^{1} embryos, GFP is expressed only in the ventral and lateral portions of each segment at stage 15 (Fig. 3H). Since in the absence of myoblast fusion most founders, marked by β-gal (Fig. 3I), also express GFP at least weakly (Fig. 3J), we conclude that cells expressing Poxm early during myogenesis will give rise to most founders of the ventral and lateral muscles. In addition, Poxm is expressed early in mesodermal cells that are not selected as progenitors, as evident from the perdurance of GFP in many mesodermal cells different from founders (Fig. 3J). Similar results were obtained for $Dmef2^{22\text{-}21}$ mutants (not shown).

Isolation and characterization of *Poxm* mutant alleles

The expression patterns of *Poxm* suggest that it plays a crucial role in myogenesis. Assuming that absence of *Poxm* functions results in lethality, we screened a collection of 1,400 lethal P-element insertions on the third chromosome (Deák et al., 1997) for lack of complementation with the deficiency $Df(3R)dsx^{D+R5}$ (see Fig. S1A in the supplementary material) (Duncan and Kaufman, 1975), which uncovers *Poxm* (Bopp et al., 1989), and subsequently for complementation with $Df(3R)dsx^{M+R29}$, whose distal break point is located proximal to *Poxm*, at 84F6-7 (Baker et al., 1991). One lethal insertion, *P282*, was identified that had inserted into the neighboring gene, 5 kb downstream of the second exon of *Poxm* (see Fig. S1A in the supplementary material). Embryos homozygous for *P282* did not show any muscle defects. Imprecise excision of this P element (Robertson et al., 1988) produced a deficiency, *Df(3R)159*, whose distal breakpoint is located about 10 kb upstream of the *Poxm* transcription start site (see Fig. S1A in the supplementary material). Its proximal breakpoint maps distal to the more proximal deficiency $Df(3R)dsx^{M+R29}$, with which it complements. Embryos homozygous for *Df(3R)159* show severe defects in the larval somatic musculature.

Since *Df(3R)159* deletes, in addition to *Poxm*, at least another gene, the observed muscle phenotype might result from the absence of more than just *Poxm* functions. Therefore, eight EMS-induced embryonic lethal mutants, obtained in a screen for genes on the third chromosome affecting neuromuscular connectivity (Sink et al., 2001; Van Vactor et al., 1993), that showed defects in muscle patterning were tested for complementation with *Df(3R)159*. One of these mutants, *R361*, failed to complement and showed the same

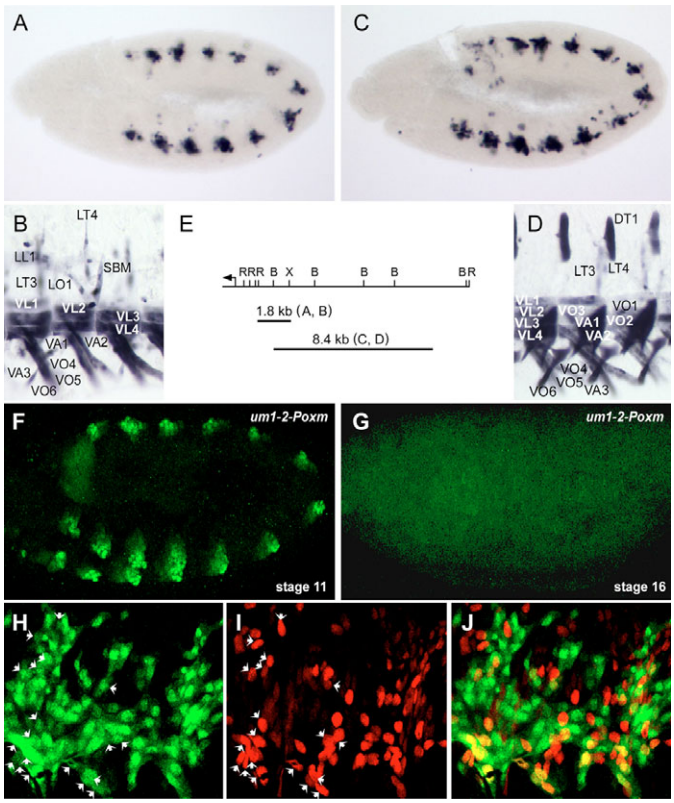


Fig. 3. Most ventral and lateral somatic muscle founders are recruited from cells expressing early Poxm. (**A-E**) Early and late Poxm expressions in the somatic mesoderm are regulated by different enhancers. Whole-mount transgenic *Poxm1.8-Gal4/UAS-lacZ* (A,B) and *Poxm8.4-Gal4/UAS-lacZ* (C,D) embryos were stained with rabbit anti-β-galactosidase antiserum. A map of the *Poxm* upstream region (B, *Bam*HI; R, *Eco*RI; X, *Xba*I), which delimits the 1.8 kb and 8.4 kb fragments used as enhancers in combination with the *hsp70* minimal promoter to drive Gal4 expression in the *Poxm-Gal4* transgenes, is shown in E. Overviews of late stage 11 embryos (A,C) and enlarged ventral and lateral views of abdominal segments A2-A4 (B) or A4-A6 (D) of stage 16 embryos are shown with anterior to the left and dorsal up. Muscle patterns (B,D) were visualized from the interior (B) or exterior (D) after staining for β-gal, by dissecting the embryos in halves along the dorsal and ventral midlines, removing tissue below the muscles, and mounting the ectoderm with the attached muscles for bright-field microscopy in a Zeiss Axiophot. The moderate to low β-gal levels observed at late stages after early activation by the 1.8 kb enhancer result from perdurance (B), whereas the high β-gal levels observed after activation by the 8.4 kb enhancer mimic late stage Poxm expression (D). For muscle nomenclature, see Bate (Bate, 1993) or Fig. 4J,N. (**F**,**G**) Absence of late Poxm expression of a *Poxm* transgene driven by the early enhancer. Homozygous $Poxm^{361}$ embryos, rescued by the *um1-2-Poxm* transgene that includes only upstream cis-regulatory sequences up to the *Xba*I site (E) and no intron, exhibit a wild-type early Poxm pattern (stage 11; F) but no late Poxm expression (stage 16; G). Confocal micrographs of embryos with their anterior to the left and dorsal side up are shown. (**H-J**) Cells expressing early Poxm give rise to most ventral and lateral muscle founders. Cells expressing early Poxm were labeled by nuclear GFP (H) and their fate was followed by confocal microscopy in *rP298-lacZ*; *Poxm1.8-Gal4/UAS-GFPnls*; lmd^{1} embryos, in which founders are marked by β-gal (I) and their fusion with FCMs is blocked. Most ventral and lateral muscle founders that are labeled by β-gal are also marked by GFP (J), many of which are marked by white arrowheads in two of the three abdominal segments of a stage 15 embryo shown in H and I.

larval muscle phenotype as *Df(3R)159*, in homozygous and transheterozygous conditions. No Poxm protein was detectable in either mutant (not shown). Sequencing of *R361* genomic DNA identified, in *Poxm*, a single point mutation, $Poxm^{R361}$, that converts a glutamine codon at position 7 of the N-terminal paired domain into an amber stop codon and hence is expected to result in a truncated N-terminal Poxm peptide of 14 amino acids (see Fig. S1B in the supplementary material). It follows that $Poxm^{R361}$ is a null allele of *Poxm*.

Loss of Poxm function causes severe disruption of the somatic muscle pattern

To investigate the effects of *Poxm* on muscle development, embryos homozygous or transheterozygous for *Df(3R)159* and $Poxm^{R361}$ were examined after visualizing their somatic muscles by staining with anti-MHC. These mutants all displayed the same severe defects in the formation of larval muscles. In our analysis, which focused on abdominal segments A2-A7, *Poxm* was considered to be required for the proper development of a specific muscle if that muscle did not form normally in a significant fraction of hemisegments in *Poxm* null mutants. It does not imply that this muscle never forms normally, as the penetrance of the phenotype may not be 100%.

In the ventral region of *Poxm* mutant embryos, usually muscles VO4-6 are absent, whereas muscles VA1-3 are still present in most segments but are poorly developed, lacking their normal shape and attachment sites (Fig. 4G,H, Fig. 5A). Further analysis revealed that muscles VL3 and VL4 are frequently abnormal or missing, whereas muscles VL1 and VL2 are occasionally or rarely affected (Fig. 5A). Also muscles VO2 and VO1 are strongly and moderately disturbed, respectively (Fig. 5A).

In the dorsolateral region, muscle DT1, in most cases, is missing or abnormal, whereas muscle DO3, which is derived from the same progenitor (Carmena et al., 1995), is mostly duplicated or abnormal and very rarely missing (Fig. 4D,E, Fig. 5A). Two additional muscles, DA3 and DO4, are occasionally abnormal, whereas the two most posterior lateral muscles, LO1 and LT4, are frequently missing and abnormal, respectively (Fig. 5A). By contrast, all dorsal muscles remain unaffected (Fig. 4A,B, Fig. 5A).

Ordering the muscles along the abscissa according to decreasing severity of their *Poxm* mutant phenotype (red bars in Fig. 5E) reveals a striking correlation with the early triangular *Poxm* expression pattern (Fig. 1D). Muscles located more ventrally or more posteriorly in a segment are always more strongly affected as compared to muscles located roughly at the same anteroposterior or dorsoventral positions, respectively (Fig. 4N). For example, muscle VL4 is affected more severely than its dorsal neighbor VL3, which is again more frequently abnormal than VL2 or VL1. Similarly, the phenotype of muscle LT4 is stronger than that of its anterior neighbors LT1-3. This phenotype suggests that it might be affected by a function that depends on a dorsoventral as well as an anteroposterior gradient, on which indeed the early *Poxm* expression pattern depends, namely on Dpp (Staehling-Hampton et al., 1994) and Wg (J.C. and M.N., unpublished), and which explains its characteristic triangular shape (Fig. 1D).

Ectopic expression of Poxm in the mesoderm generates additional muscles

To test whether Poxm can determine muscle development, we expressed it ectopically and analyzed its effect on myogenesis. *24BGal4* was used to drive expression of *UAS-Poxm* in the entire mesoderm beginning at mid stage 10 (Michelson, 1994). Ectopic Poxm produces a severely altered muscle pattern, which varies

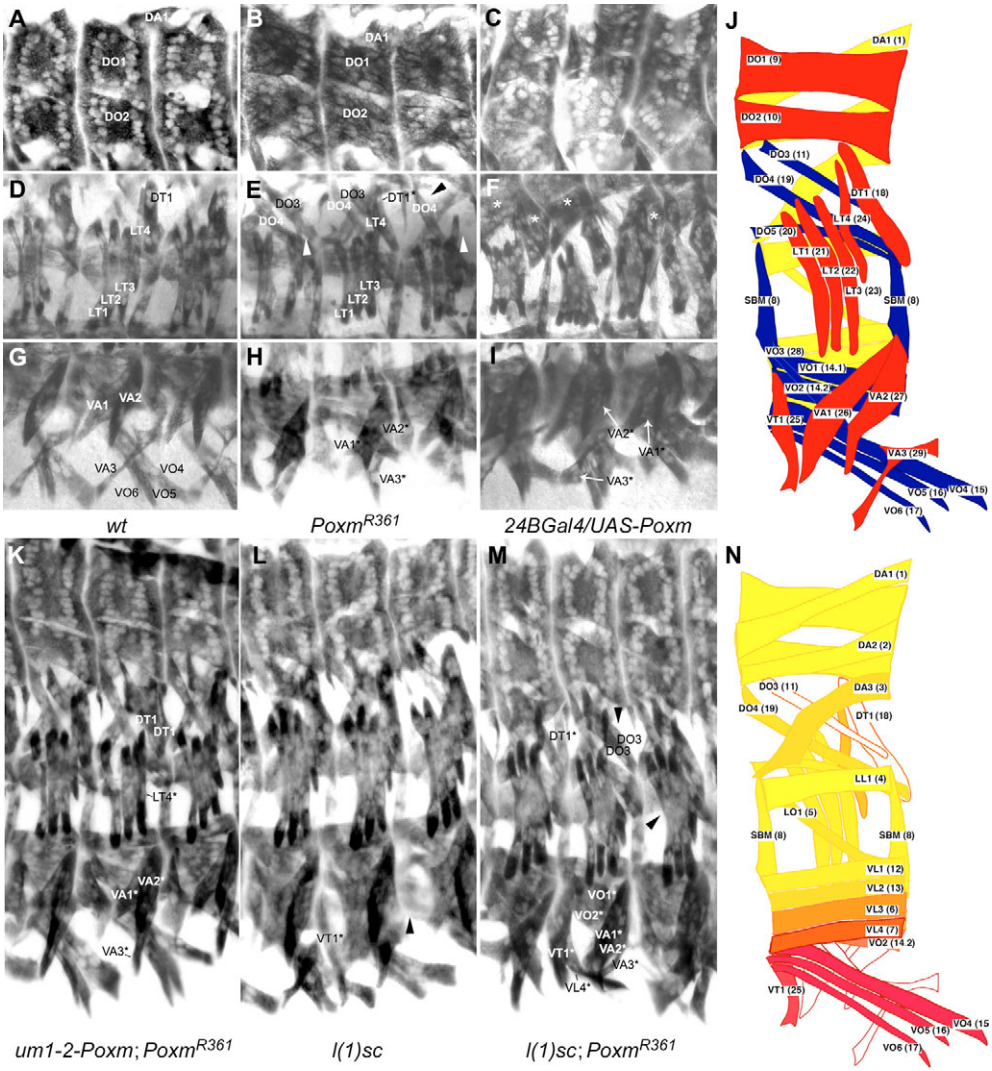


Fig. 4. Muscle phenotypes of *Poxm* and *l(1)sc* mutants. (**A-I**) Muscle phenotypes resulting from loss of, or ectopic, Poxm. Dorsal (A-C), lateral (D-F) and ventral (G-I) muscles were visualized using an anti-MHC antiserum in three abdominal hemisegments of stage 16 wild-type (A,D,G), *Poxm*^{R361} (B,E,H), and *24BGal4/UAS-Poxm* (C,F,I) embryos, oriented with anterior to the left and dorsal up. In *Poxm*^{R361} mutants, positions of missing muscles DT1 (white arrowheads) or an abnormal muscle DT1 (*) and of a missing muscle DO3 (black arrowhead) are indicated in E, ventral muscles VO4-6 are missing (H), and ventral muscles VA1-3* have lost their normal shape and attachments (H), as evident from a comparison with the wild-type ventral muscle pattern (G). Muscle VL4 is also frequently absent, as evident from inspection of a plane of focus interior to and below that shown in H. A detailed analysis of the *Poxm*^{R361} muscle phenotype is summarized in Fig. 5A. Ectopic ubiquitous mesodermal expression of Poxm (C,F,I) generates ectopic dorsal and lateral muscles (marked by asterisks in F) and enlarges some ventral muscles (arrows in I), the number and positions of which are not altered. (**J**) Schematic external view (dorsal up and anterior to the left) of larval muscles in abdominal segments A2-A7 (Ruiz-Gómez et al., 1997), with external muscles in red and more internal muscles in blue and yellow; muscles are designated and numbered according to Bate (Bate, 1993) and in parentheses according to Crossley (Crossley, 1978). (**K-M**) Muscle phenotypes of *Poxm* mutants rescued by early Poxm, and of *l(1)sc*; *Poxm* double and *l(1)sc* single mutants. Muscle phenotypes were visualized using an anti-MHC antiserum in three abdominal hemisegments of *Poxm*^{R361} embryos rescued by two copies of the *um1-2-Poxm* transgene (K), of *Df(1)sc*^{19} embryos (L), and of *Df(1)sc*^{19}; *Poxm*^{R361} embryos (M) at stage 16. Anterior is to the left and dorsal up. A detailed analysis of these phenotypes is summarized in Fig. 5B-D. Some muscles that are abnormal in shape and/or position are marked by asterisks, duplicated muscles DT1 (K) and DO3 (M) are labeled, and missing muscles VT1 (L), DT1 and LO1 (M) are indicated by black arrowheads. Ventral muscles VO4-6 that are missing in nearly all segments of *Poxm* single or double mutants (Fig. 5A,D) are also absent but not marked (M). (**N**) Schematic internal view of a hemisegment opposite to that shown in J (Ruiz-Gómez et al., 1997) of the muscle phenotype attributable to the absence of the early *Poxm* function, in which each muscle is colored in a graded fashion from red (0%) to yellow (100%) corresponding to the fraction of normal muscles observed (Fig. 5A). Muscles DO3, DT1 and VA1-3 are not colored as the contribution to their phenotype of the missing early Poxm cannot be estimated because they are also affected by the late *Poxm* function, and muscle VO3 is not colored because it has not been recorded in Fig. 5A.

among different segments and embryos. The most striking defects occur in the dorsal and dorsolateral muscles, where Poxm is normally absent or present at low levels (Fig. 4C,F). In the dorsal region, which includes four muscles in wild-type embryos (Bate, 1993) (Fig. 4A,J), ectopic muscles are generated in most segments (Fig. 4C). Ectopic muscles similar in shape and orientation to muscle DA3 occupy the dorsolateral region (Fig. 4C,F), which is largely free of muscles in wild-type embryos (Fig. 4D). Usually several muscles with abnormal shape occur at the position of muscle DT1 (Fig. 4F), whereas muscles LL1, DO4 and DO5 exhibit aberrant shapes or are missing in some segments. In addition, some of the lateral muscles are abnormally shaped. By contrast, the ventral muscles, all of which exhibited a strong early Poxm expression (Fig. 1C,D), remain largely unaffected, although some muscle fibers appear enlarged (Fig. 4I).

Poxm regulates the formation of adult muscle precursors

As adult muscle precursors derive from progenitors that also generate founders of larval muscles (Bate et al., 1991; Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997), we suspected that *Poxm* also affects adult muscle precursors. To test this

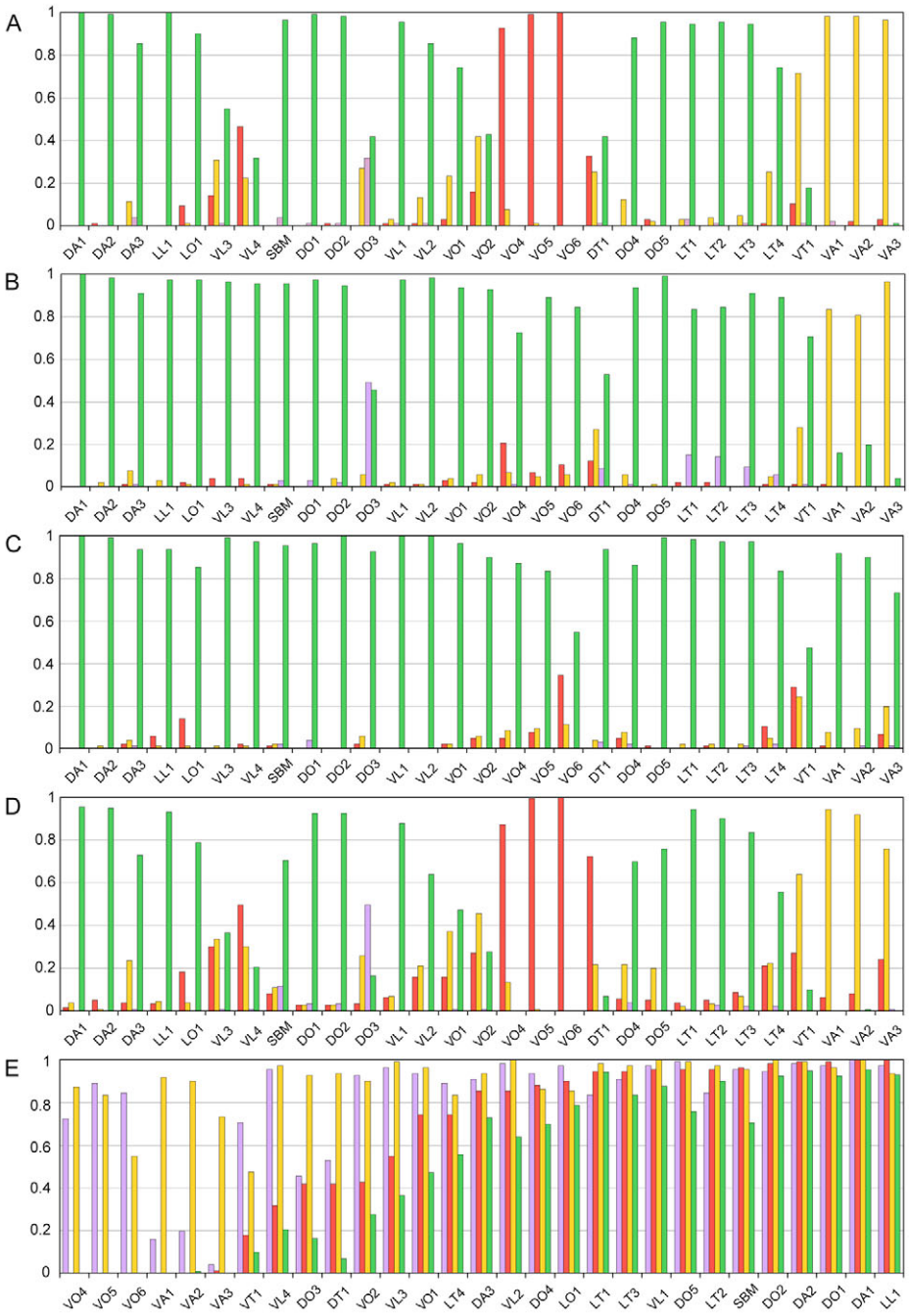
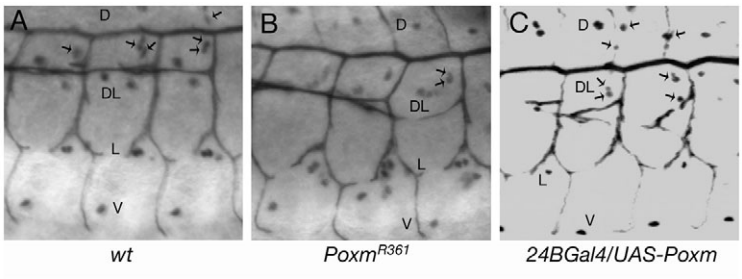


Fig. 5. Larval body wall muscle phenotypes of *Poxm* and *l(1)sc* single and double mutants, and of *Poxm* mutants rescued by early Poxm. (**A-D**) The somatic muscle patterns of $Poxm^{R361}$ embryos (A), $Poxm^{R361}$ embryos rescued by two copies of the *um1-2-Poxm* transgene (B), of $Df(1)sc^{19}/Df(1)sc^{19}$ or *Y* (C), and $Df(1)sc^{19}/Df(1)sc^{19}$ or *Y*; $Poxm^{R361}$ (D) embryos and of *y w* control embryos (not shown) were analyzed at stage 16 under bright-field optics in a Zeiss Axioplan 2 microscope after staining with anti-MHC antiserum, dissection along the ventral midline, and removal of internal tissues. Each muscle plotted on the abscissa was scored for absence (red), abnormality (yellow), duplication (purple), or wild-type appearance (green) in each of 108 (A-C) or 168 (D) hemisegments in abdominal segments A2-A7, and the resulting fractions were plotted on the ordinate. Muscles of the *y w* control embryos were usually normal in all 108 hemisegments scored, with the occasional absence, duplication or abnormality of a single muscle. (**E**) The fractions of normal muscles shown in A-D are plotted for *Poxm* mutants rescued by early Poxm (purple), *Poxm* (red) and *l(1)sc* (yellow) single mutants, and for *l(1)sc*; *Poxm* (green) double mutants, whereas muscles are ordered along the abscissa with decreasing abnormality of *Poxm* mutant phenotypes.

hypothesis, we analyzed the effects of loss-of-function and ectopic expression of *Poxm* on the expression of Twi, which is present in all adult muscle precursors but not in larval founders after germ band retraction (Bate et al., 1991). In stage 14 wild-type embryos, adult muscle precursors appear in four groups with a single precursor each in the ventral and dorsal groups (VaP and DaP) and two each in the dorsolateral and lateral groups (DLaPs and LaPs) (Fig. 6A) (Bate et al., 1991; Ruiz Gómez and Bate, 1997).

In the lateral mesoderm of embryos expressing Poxm ubiquitously, in most segments DLaPs are missing and only one of the two LaPs is present (Fig. 6C,D). The reverse situation was found in *Poxm* mutants (Fig. 6B,D). The number of LaPs increases to four to seven cells in each abdominal hemisegment, and more than two DLaPs are present in 20% of the segments. Therefore, in the lateral portions of the abdominal segments, Poxm acts to prevent the formation of supernumerary adult muscle precursors and, when ectopically expressed, can inhibit the formation of normal adult muscle precursors.

In the dorsal region, after mesodermal ubiquitous expression of Poxm, on average two DaPs instead of one are present in about half of the segments (Fig. 6C,D). This result correlates with the appearance of ectopic dorsal muscles (Fig. 4C) and hence suggests that ectopic expression of Poxm leads to the production of supernumerary adult muscle precursors and muscle founders in the region where normally only a very low level of Poxm is expressed at early embryonic stages. In embryos lacking Poxm, however, DaPs remain largely unaffected (Fig. 6B,D).



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Genotype	Number of Hemisegments	DaP	DLaP	LaP	VaP
Poxm^{R361}	90	94 (90)	204 (180)	518 (180)	96 (90)
24B>Poxm	90	130 (90)	31 (180)	105 (180)	92 (90)

Fig. 6. Loss of Poxm and ectopic Poxm change the number of adult muscle precursors. (**A-C**) Expression of Twi in abdominal hemisegments of wild-type (A), *Poxm*^{R361} (B), and *24BGal4/UAS-Poxm* (C) stage 14 embryos was visualized by staining with an anti-Twi antiserum (anterior to the left and dorsal up). Only precursors of adult muscles and the alary cells express Twi at this stage [alary cells, marked by black arrows, are located on the segment margins in the dorsal and dorsolateral region; see Bate et al. (Bate et al., 1991)]. The adult muscle precursors are arranged in dorsal (D), dorsolateral (DL), lateral (L) and ventral (V) groups. The staining of the trachea is caused by a crossreactivity of the anti-Twi antiserum. (**D**) Analysis of number of adult muscle precursors in embryos mutant for *Poxm* or expressing ubiquitous mesodermal Poxm. The number of adult muscle precursors was analyzed in 90 hemisegments each of *Poxm*^{R361} and *24BGal4/UAS-Poxm* embryos. The total numbers for 90 hemisegments are shown for the dorsal (DaP), dorsolateral (DLaP), lateral (LaP) and ventral (VaP) group of adult muscle precursors with the numbers expected for wild-type embryos in parentheses.

In the ventral region, the number of VaPs is hardly changed not only in the presence of mesodermal ubiquitous Poxm but also in the absence of Poxm (Fig. 6B-D).

Poxm acts upstream of the muscle identity gene *slou*

Early expression of *slou*, one of the well-studied muscle identity genes (Dohrmann et al., 1990; Knirr et al., 1999; Ruiz-Gómez et al., 1997), occurs in a subset of muscle progenitors and their offspring founders, some of which also express Poxm (Fig. 1H). This raises the possibility of an epistatic relationship between these genes. Early Slou-expressing cells are arranged in three groups of muscle founders (Fig. 7A): group I will generate muscles LO1 and VT1; group II, muscles VA1-3 and the VaP; and group III, muscles DO3 and DT1 (Carmena et al., 1995; Dohrmann et al., 1990). After stage 13, Slou remains expressed only in the precursors of muscles DT1, VT1 and VA2 (Carmena et al., 1995; Dohrmann et al., 1990) (Fig. 7C,E), two of which, DT1 and VA2, also express Poxm (Fig. 1J,L). In *Poxm*^{R361} embryos, Slou protein is expressed in groups I and II, yet is absent from group III in most, though occasionally observed in more posterior, abdominal segments during late stage 12 (Fig. 7B). After stage 13, Slou is detectable only in the precursor of muscle VT1 but no longer maintained in that of VA2 in abdominal segments (Fig. 7D). Therefore, Poxm is essential for the activation of *slou* in the progenitor of muscle DT1 and for its maintenance in the precursor of muscle VA2.

In *24BGal4/UAS-Poxm* embryos, in which Poxm is ubiquitously expressed in the mesoderm, additional muscles expressing Slou were found in the dorsolateral portion of some segments (Fig. 7F), which suggests that in these cells ectopic Poxm suffices to activate *slou* and corroborates the observation that *Poxm* acts upstream of the muscle identity gene *slou*.

Early Poxm largely rescues the muscle phenotype of *Poxm* mutants

Since Poxm is expressed during early myogenesis in cells that later give rise to progenitors of most of the ventral and lateral muscles, it may play an important role in the initiation of muscle patterning. To investigate which part of the *Poxm*^{R361} muscle phenotype results from the loss of this early *Poxm* function, a transgene expressing *Poxm* only during the early myogenic stages (Fig. 3F,G), *um1-2-Poxm*, was introduced into *Poxm*^{R361} embryos. In these embryos, the phenotypes of muscles VO4-6, VL2-VL4, VO2, VO1, LO1, LT4 and VT1 are efficiently rescued (Fig. 4K; Fig. 5B,E). The only muscles affected in *Poxm* mutants (Fig. 5A) that are only slightly rescued by early Poxm (Fig. 5B) are DT1, DO3 and VA1-3, in which Poxm is also expressed during later stages in their founders and/or muscle precursors (Fig. 4K; Fig. 5B,E). These results strongly suggest that *Poxm* exerts an early function, demarcating a mesodermal domain of competence for ventral, lateral and dorsolateral somatic muscle development.

Partial redundancy of early *Poxm* and *l(1)sc* functions in somatic myogenesis

The partial penetrance of the *Poxm* muscle phenotype (Fig. 5A) suggests that the early *Poxm* function is largely redundant with that of other genes, an argument also raised to explain the weak muscle phenotype of *l(1)sc* mutants (Carmena et al., 1995). The *l(1)sc* gene encodes a bHLH transcription factor the function of which is thought to be required for the selection of muscle progenitors (Baylies et al., 1998; Carmena et al., 1995). Therefore, we examined the effect of *Poxm* and *l(1)sc* mutations on larval muscle development in single and double mutant embryos (Fig. 5A,C,D).

In agreement with earlier studies (Carmena et al., 1995), *l(1)sc* mutants exhibit a weak muscle phenotype, which deviates only slightly from that of wild-type embryos (Fig. 4L, Fig. 5C). Although

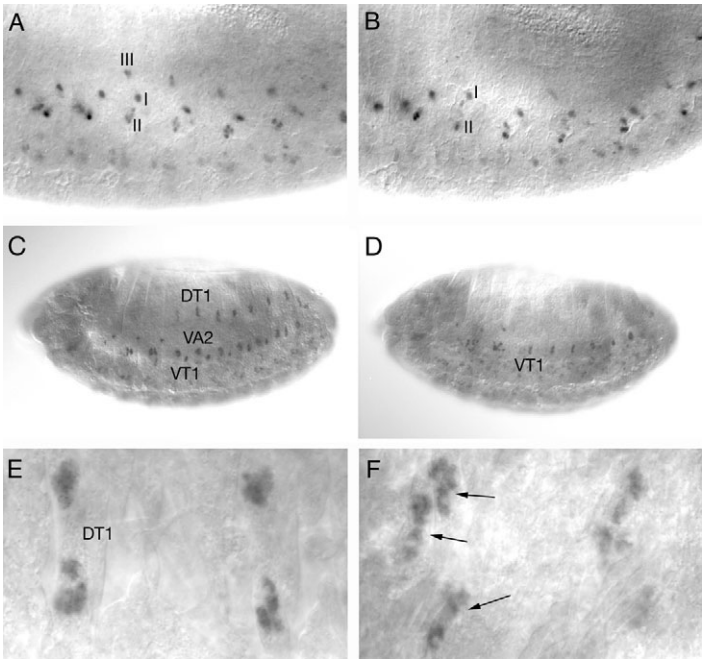

Fig. 7. Altered Slou expression in *Poxm* mutants or in the presence of ectopic Poxm. Slou expression in wild-type (**A**,**C**,**E**), $Poxm^{R361}$ (**B**,**D**), and *24BGal4/UAS-Poxm* (**F**) embryos at stage 12 (A,B), 14 (C,D) and 16 (E,F) was visualized by staining with an anti-Slou antiserum. Ventrolateral views of whole embryos (C,D) and enlarged ventrolateral (A,B) and dorsolateral (E,F) views of parasegments 4-10 (A,B) and 6-7 (E,F) are shown with anterior to the left and dorsal up. At late stage 12, Slou is expressed in three groups, I-III, of muscle founder cells of each abdominal segment of wild-type embryos (A), whereas it is expressed only in groups I and II of $Poxm^{R361}$ mutants (B). At stage 14, Slou is expressed in the precursors of dorsal muscle DT1 and of ventral muscles VT1 and VA2 of each abdominal segment of wild-type embryos (C), whereas it is detectable only in the precursor of ventral muscle VT1, but not of muscles DT1 and VA2, in $Poxm^{R361}$ embryos (D). After ectopic expression of Poxm, additional muscles express Slou (arrows in F) in a dorsolateral region where Slou is expressed only in muscle DT1 of stage 16 wild-type embryos (E).

$Poxm^{R361}$ embryos show a considerably stronger muscle phenotype, most lateral and dorsal muscles are normal (Fig. 5A). Assuming that *Poxm* and *l(1)sc* act independently in muscle development, we expect that the probability of a muscle being wild-type in *Df(1) l(1)sc*^{19}*/Y*; $Poxm^{R361}$ embryos is the product of the probabilities of the muscle being wild-type in the single mutants. Conversely, if we find significantly enhanced probabilities for muscle defects in double mutants, we may conclude that *Poxm* and *l(1)sc* exhibit partially redundant functions during muscle development. Applying this test to the results summarized in Fig. 5A,C,D, we find that most muscles are affected independently or nearly independently, with some notable exceptions. These concern muscles VL1-3, SBM, VO1, VO2, DT1, LT3, LT4 and VA3 that are more often absent. Some muscles are strongly affected in *Poxm* null mutants, such as muscles VO4-6 or muscles VA1-3. Among the other muscles, the more ventral and the more posterior a muscle is located within a segment, the more probable it is that it will show an enhanced phenotype in double mutants (Fig. 5E). Clearly, there is some redundancy between *Poxm* and *l(1)sc* functions in the somatic mesoderm, which is restricted largely to ventral and posterior muscles.

Late *Poxm* function specifies muscle identity

In *Poxm* mutants, only muscle DO3 is frequently duplicated (Fig. 5A). This duplication results from the transformation of muscle DT1 to DO3, as previously observed for muscles derived from the same progenitor in the absence of a muscle identity gene that is asymmetrically expressed in the two founders and muscle precursors (Knirr et al., 1999; Ruiz-Gómez et al., 1997). Thus, late expression of *Poxm* in the precursor of muscle DT1, but not of DO3, is crucial for their distinction and hence serves a muscle identity function. However, a more detailed analysis shows that muscle DT1 is missing in only two thirds (23/34) of all cases in which muscle DO3 is duplicated (see Table S1 in the supplementary material). In the remaining 11 cases, muscle DT1 is normal (4), abnormal (6) or duplicated (1). This finding suggests that the late *Poxm* function is necessary in about 10% (11/108) of all cases to prevent an additional division that generates a second founder of muscle DO3.

Absence of Poxm in their founders results in abnormal muscles VA1-3 (Fig. 5A) that cannot be rescued by the early Poxm function (Fig. 5B), which suggests that their proper specification also depends on the late function of *Poxm*.

DISCUSSION

Our results demonstrate that the development of larval body wall muscles depends on distinct *Poxm* functions during two phases. The early function of *Poxm* specifies, within the high Twi or Slp domain, a subdomain of competence for lateral and ventral muscle development, the 'Poxm competence domain' (Fig. 8). This function appears to be analogous to that of *tin*, which specifies competence for heart and dorsal muscle development in the complementary part of the Slp domain (Azpiazu and Frasch, 1993; Michelson et al., 1998; Yin and Frasch, 1998). *Poxm* and *tin* thus subdivide the posterior Slp domain into ventral and dorsal subdomains in a manner similar to the partitioning by *serpent* and *bap* of the anterior Eve domain into the ventral fat body and the dorsal visceral mesoderm anlagen (Azpiazu et al., 1996; Riechmann et al., 1998). After selection of muscle progenitors, proper development of a few muscles still depends on *Poxm*, which is expressed in muscles DT1 and VA1-3. This late function of *Poxm* participates in founder specification and muscle differentiation, as is characteristic for muscle identity genes. Finally, our findings suggest a solution to a conceptual problem of the current model of somatic myogenesis, the *l(1)sc* conundrum.

Early Poxm specifies competence for somatic myogenesis in partial redundancy with similar functions of L(1)sc

The muscle phenotype of *Poxm* mutant embryos and its rescue by early Poxm expression shows that the early *Poxm* function is crucial for the proper development of many ventral and lateral muscles (Fig. 5A,B). In addition, the generation of ectopic dorsal and dorsolateral muscles by ectopic Poxm suggests that *Poxm* has the ability to change cell fates and render cells competent for myogenesis. Therefore, we propose that early Poxm demarcates a ventral and lateral domain of competence for somatic myogenesis.

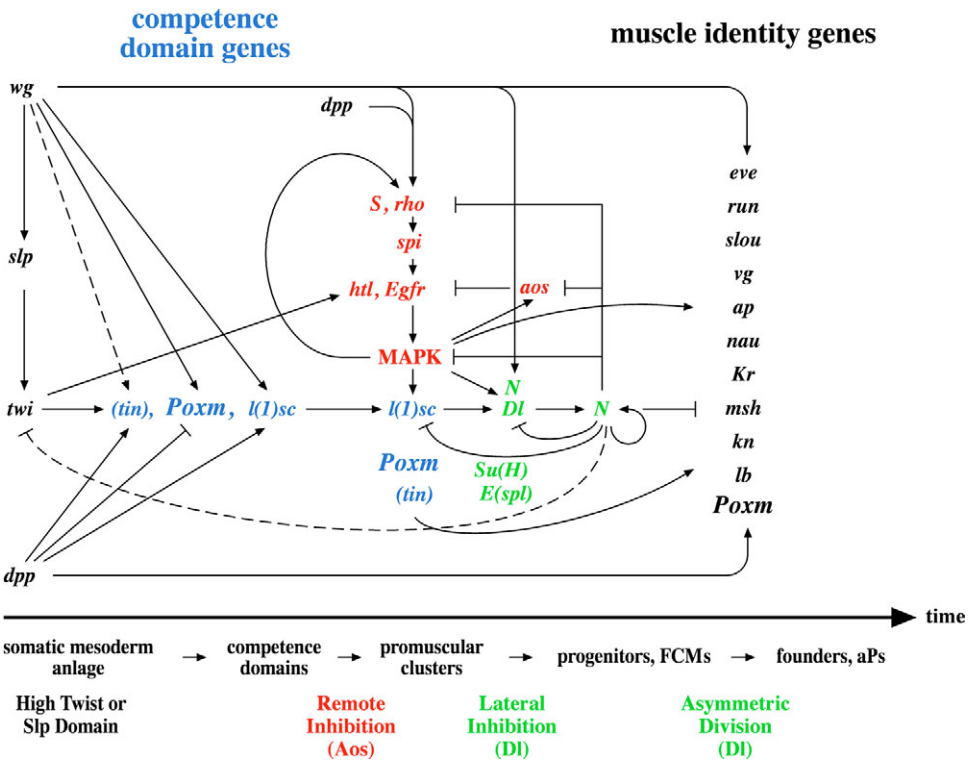

Fig. 8. Regulatory network of somatic myogenesis. The scheme depicts the interactions among the major genes and/or their products that regulate the development of larval body wall muscles from gastrulation to the specification of muscle founder cells, as explained in detail in the text. After the establishment of the high Twi domain in the mesoderm underneath the posterior portions of parasegments by Slp, domains competent for somatic myogenesis are specified by 'competence domain genes', such as *l(1)sc*, *tin* (the hypothetical role of *tin* as competence domain gene is indicated by parentheses) and *Poxm*, regulated by Twi and the ectodermal signals Wg and Dpp. These signals, in combination with the localized EGF (Spi) and FGF (Pyr, Ths) signals and through remote inhibition of Spi signaling by Aos (Freeman, 1997), determine the promuscular clusters, which express *l(1)sc* and activate the MAPK signaling pathway. Singling out of muscle progenitors and separation from fusion-competent myoblasts (FCMs) occurs by lateral inhibition, which is mediated by N signaling that is coupled to MAPK signaling through multiple feedback loops. At this stage, or subsequently, in muscle founder cells generated from progenitors by asymmetric division mediated by N signaling, muscle identity genes, such as *Poxm*, *Kr* and *slou*, are activated by the integration on their enhancers of competence domain gene products with the effectors of the mentioned signals (for references, see text). Hypothetical interactions are indicated by dashed lines.

The partial penetrance of the *Poxm* mutant phenotype implies the existence of other competence domain genes performing partially redundant functions. We have shown that Poxm and L(1)sc partially co-localize in the promuscular clusters and muscle progenitors (Fig. 2G-I). In addition, a detailed analysis of *l(1)sc* and *Poxm* single and double mutants demonstrates that their functions are partially redundant (Fig. 5A,C,D). Since the muscle phenotype of *l(1)sc*; *Poxm* double mutants still shows partial penetrance (Fig. 5D), additional competence domain genes should be expressed in the Slp domain. One of them is probably *tin*, which is initially expressed in the entire early mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990), because *tin* mutants affect muscle development in the dorsal as well as lateral and ventral Slp domain (Azpiazu and Frasch, 1993; Michelson et al., 1998). Another candidate is *D-six4*, which is required for the development of specific muscles that arise from the dorsolateral and ventral regions (Clark et al., 2006).

Thus, after the initial subdivision of the mesoderm, the high Twi domain is further subdivided by competence domain genes (Fig. 8), which specify domains that become competent to select progenitors of distinct subsets of somatic muscles and/or of myocardial and pericardial cells by responding to spatially restricted extracellular signals (Azpiazu and Frasch, 1993; Halfon et al., 2000). These competence domain genes act in a cooperative manner to determine the identities of specific muscles by regulating the expression of the muscle identity genes. When one of them is inactivated, in some cells active competence domain genes can partially compensate for the inactive gene by activating its target genes such that these sometimes, but not always, exceed the threshold levels required for normal development. Hence, muscles derived from these cells exhibit a mutant phenotype with partial penetrance. For other cells, active competence domain genes can compensate completely for the missing gene activity such that these cells will adopt the proper fate and the muscles develop normally. This illustrates that competence is not a matter of 'all' or 'nothing' for muscle development. The deeper reason for this, we deem, is that the genetic program regulating myogenesis is not organized in a hierarchical fashion but rather as a complex gene network (Fig. 8) that has an integrated function which is much more stable against mutations within the network than a hierarchical regulation would be.

Poxm is a muscle identity gene activating the muscle identity gene *slou*

Muscle identity genes usually encode transcription factors, such as Slou, Nau, Ap, Vg, Kr, Eve, Msh, Lb, Run and Kn (Bate et al., 1993; Bourgouin et al., 1992; Carmena et al., 2002; Dohrmann et al., 1990; Frasch et al., 1987; Jagla et al., 2002; Michelson et al., 1990; Knirr et al., 1999; Nose et al., 1998; Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997), that are expressed in subsets of

muscle progenitors and founders and determine in a combinatorial fashion the identity of each muscle founder and its subsequent differentiation into a specific muscle of defined size, shape, attachment sites, and innervation (Baylies et al., 1998; Dohrmann et al., 1990; Ruiz-Gómez et al., 1997). We envision the activation of these genes in promuscular clusters or, after lateral inhibition, in muscle progenitors (Carmena et al., 1995; Carmena et al., 1998a) by Twi and/or the products of competence domain genes and through combinations of localized extracellular signals from the ectoderm and mesoderm (Azpiazu and Frasch, 1993; Halfon et al., 2000). During asymmetric division of progenitors, expression of a muscle identity gene may be maintained in one or both of the two sibling founders, or it may persist in the founder when division generates a founder and an adult muscle precursor. Late expression of Poxm illustrates all three cases. It is expressed in progenitors P26/27 and P29/VaP, which are derived from promuscular cluster 10 and give rise to the founders of muscles VA1 (F26) and VA2 (F27), and to the founder of muscle VA3 (F29) and the ventral adult precursor VaP (Carmena et al., 1995; Dohrmann et al., 1990). Poxm is also expressed in the progenitor derived from cluster 13, P11/18, which generates the founders of muscles DO3 (F11) and DT1 (F18). Although Poxm expression persists in F29 and F18 but not in their siblings, it is maintained in both sibling founders F26 and F27.

The late function of *Poxm* is identified as a muscle identity function by the high correlation between absence of muscle DT1 and corresponding duplication of muscle DO3 in *Poxm* mutants (Fig. 5A and see Table S1 in the supplementary material). If Poxm was the sole determinant discriminating between F11 and F18, mesodermal ubiquitous expression of Poxm would be expected to transform muscle DO3 into DT1. Our results confirm the presence of additional muscles in the region of muscle DT1. It is possible that one of these originates from a transformed F11, but it is impossible to tell whether muscle DO3 is missing (Fig. 4F) because additional muscles have been recruited.

It has been shown that in the process of muscle diversification, identity genes may repress or activate other identity genes in progenitors and founders (Jagla et al., 1998; Jagla et al., 2002; Knirr et al., 1999; Nose et al., 1998; Ruiz-Gómez et al., 1997). We found that the muscle identity gene *slou* fails to be activated in P11/18 of *Poxm* mutants. The simplest explanation of this result is that activation and maintenance of *slou* expression depend on Poxm in P11/18 and its offspring founders. In addition, *slou* expression is not maintained in F27 of *Poxm* mutants despite its initial activation in P26/27. It therefore appears that in P26/27 and its offspring F26 and F27, in addition to Kruppel (Kr) (Ruiz-Gómez et al., 1997), Poxm is necessary for the maintenance of *slou* expression. Although Poxm expression is maintained in both F26 and F27, *slou* expression is restricted to F27 because *Kr* is repressed in F26 by N signaling. Apparently, Kr is the crucial determinant that distinguishes F26 from F27, as F27 is altered to F26 in *Kr* or *numb* mutants (Ruiz Gómez and Bate, 1997; Ruiz-Gómez et al., 1997).

As *Poxm* is expressed in both F26 and F27, whereas its expression is restricted to F18 and not maintained in F11, its late expression in F26 and F27 must be regulated differently from that in F11 and F18 where it appears to be subject to asymmetric N signaling (Ruiz Gómez and Bate, 1997) repressing *Poxm* in F11.

These considerations imply that *slou* is part of the same gene network as *Poxm*, a conclusion consistent with our gene network hypothesis since, in the first test of this hypothesis, *slou* had been isolated as a PRD 9 gene on the basis of its homology to the *prd* gene (Frigerio et al., 1986).

A solution of the *l(1)sc* conundrum

The mechanism of progenitor selection from the somatic mesoderm depends on a process of lateral inhibition very similar to that of neuroblast or sensory organ precursor (SOP) selection in the neuroectoderm from proneural clusters expressing the proneural genes (Bate et al., 1993; Corbin et al., 1991). Because of this similarity, a search among proneural genes for 'promuscular' genes expressed in the somatic mesoderm was performed (Carmena et al., 1995). This search identified a single proneural gene, *l(1)sc*, a member of the *achaete-scute* complex (AS-C), that is expressed in promuscular clusters of the somatic mesoderm. It was, therefore, attractive to consider its function in myogenesis to be analogous to that of proneural genes in neurogenesis (Carmena et al., 1995; Carmena et al., 1998a). However, whereas proneural genes confer on neuroectodermal cells the ability to become neural precursors rather than epidermal cells, which is their default fate (Campuzano and Modolell, 1992), *l(1)sc* does not seem to confer on mesodermal cells the ability to undergo somatic myogenesis instead of becoming part of the visceral mesoderm, heart or fat body. When L(1)sc was expressed in the entire mesoderm from stage 8 onward, other mesodermal tissues could not be transformed into somatic mesoderm (Carmena et al., 1995), whereas a deficiency of *l(1)sc* resulted in only minor defects of somatic muscle development (Fig. 5C) (Carmena et al., 1995). In addition, as the *l(1)sc* muscle mutant phenotype can be rescued by ubiquitous mesodermal L(1)sc expression (Carmena et al., 1995), its expression in clusters is not decisive for the formation of promuscular clusters and, therefore, *l(1)sc* cannot play the decisive role in the development of larval body wall muscles that has been proposed (Carmena et al., 1995). Thus, although *l(1)sc* serves as an excellent marker for promuscular clusters, it lacks a property expected to be crucial for a promuscular gene. Are there genes that might qualify as promuscular genes and thus extend the close evolutionary relationship of progenitor selection between myogenesis and neurogenesis (Jan and Jan, 1993)?

There is indeed a gene that is homologous to proneural genes and expressed in the somatic mesoderm, in the absence of which somatic myogenesis is seriously disturbed. This gene is *twi*, whose function at stages 10 and 11 more closely corresponds to that of a promuscular gene and which, like *l(1)sc*, encodes a bHLH transcription factor. Although Twi is also expressed earlier when it is required for mesoderm specification during gastrulation, this early function can be distinguished from its later 'promuscular' function in temperature-sensitive mutants (Baylies and Bate, 1996). In these mutants, only high levels of Twi activity, necessary for the formation of the somatic mesoderm, are abolished and no normal somatic muscles develop (Baylies and Bate, 1996). Moreover, ubiquitous expression of high levels of Twi in the mesoderm blocks all other mesodermal fates, transforming them to somatic mesoderm (Castanon et al., 2001). Since the subsequent patterning of somatic muscles depends critically on the relative levels of the products of *twi* and the proneural gene *da* (Castanon et al., 2001), it seems appropriate to consider them both as promuscular genes.

In addition to its strict requirement for somatic myogenesis, the proposed promuscular function of *twi* may be subject to lateral inhibition by N signaling, in further analogy to proneural functions in neurogenesis. This is apparent from experiments demonstrating that the restriction of high Twi levels to the Slp domain during stage 9 depends on N signaling (Brennan et al., 1999; Tapanes-Castillo and Baylies, 2004), which downregulates *twi* in the mesoderm underlying the anterior regions of parasegments where Slp does not override it (Riechmann et al., 1997). Since this process acts directly

on an identified *twi* enhancer during stages 9 and 10 (Tapanes-Castillo and Baylies, 2004), it is conceivable that this enhancer also responds to N signaling during the subsequent lateral inhibition. An alternative, though not mutually exclusive, mechanism for the downregulation of *twi* implicates the Gli-related zinc finger transcription factor Lmd (Minc), whose expression is maintained by N signaling and in the absence of which *twi* is not downregulated in fusion-competent myoblasts (Duan et al., 2001; Ruiz-Gómez et al., 2002).

During lateral inhibition, loss of Twi precedes that of L(1)sc in the promuscular clusters (Carmena et al., 1995). It is therefore possible that *l(1)sc* expression in these cells also depends on high levels of Twi, i.e. on Twi homodimers (Fig. 8). Consistent with this interpretation, shifting the equilibrium between Twi homodimers and Twi-Da heterodimers in favor of the latter represses *l(1)sc* (Castanon et al., 2001). Since early Poxm expression also depends on Twi (J.C. and M.N., unpublished), *Poxm* would be similarly repressed in promuscular clusters through lateral inhibition, either indirectly by repression of *twi* and/or directly by Twi/Da heterodimers. Such a mechanism might apply generally to both competence domain genes and muscle identity genes during lateral inhibition of promuscular clusters.

Thus, *twi* satisfies two criteria considered to be crucial for a promuscular gene in analogy to those of proneural genes in neurogenesis. However, a third criterion is not fulfilled by *twi*: its expression, in contrast to that of proneural genes in the neuroectoderm, is ubiquitous rather than restricted to promuscular clusters although this criterion is not a crucial property of proneural genes (Rodríguez et al., 1990). Yet promuscular clusters from which the myogenic progenitors are selected exist, as evident from the pattern of *l(1)sc* expression (Carmena et al., 1995). These promuscular clusters depend on combinations of the long-range ectodermal signals Wg and Dpp (Lee and Frasch, 2000; Carmena et al., 1998a) and the localized activities of the EGF signal Spi in the mesoderm and the FGF signals Pyr and Ths in the ectoderm (Buff et al., 1998; Carmena et al., 1998a; Carmena et al., 2002; Michelson et al., 1998; Stathopoulos et al., 2004). These signals, together with Twi and/or products of competence domain genes depending on Twi, determine the promuscular clusters by activating specific combinations of muscle identity genes (Halfon et al., 2000) (Fig. 8). The identity of the promuscular clusters depends not only on the combination of these signals but, in the case of MAPK signaling elicited by FGF and/or EGF, also on their intensity (Buff et al., 1998). In addition, multiple positive and negative feedback loops of the coupled MAPK and N signaling networks ensure a stable selection and specification of muscle progenitors not only within, but also beyond, the limits of a promuscular cluster (Carmena et al., 1998a; Carmena et al., 2002). Such a conclusion implies that these clusters are not a priori determined, but depend on the range and intensities of the MAPK activating signals, in agreement with our assumption that it is not the expression of *l(1)sc* that determines the promuscular clusters. In fact, it may be the absence of such a priori determined clusters of equivalent cells in the somatic mesoderm that necessitates such a complex N and Ras signaling circuitry (Fig. 8).

Therefore, we propose that *twi and da*, instead of *l(1)sc*, function as promuscular genes by regulating the activities of competence domain genes, which in turn regulate the combinatorial activities of muscle identity genes and thereby specify the fates of muscle progenitors and founders (Fig. 8). It is nevertheless surprising that *l(1)sc* appears to be expressed in all promuscular clusters even though its function is not necessary in most of them. It is possible that this expression pattern is an evolutionary remnant of an atavistic promuscular function of *l(1)sc* that was later replaced by the promuscular function of *twi* on whose expression *l(1)sc* activity depends.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/22/3985/DC1

References

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev.* **7**, 1325-1340.

Azpiazu, N., Lawrence, P. A., Vincent, J.-P. and Frasch, M. (1996). Segmentation and specification of the *Drosophila* mesoderm. *Genes Dev.* **10**, 3183-3194.

Baker, B. S. and Wolfner, M. F. (1988). A molecular analysis of *doublesex*, a bifunctional gene that controls both male and female sexual differentiation in *Drosophila melanogaster*. *Genes Dev.* **2**, 477-489.

Baker, B. S., Hoff, G., Kaufman, T. C., Wolfner, M. F. and Hazelrigg, T. (1991). The *doublesex* locus of *Drosophila melanogaster* and its flanking regions: a cytogenetic analysis. *Genetics* **127**, 125-138.

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila*. *Development* **110**, 791-804.

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster*, Vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 1013-1090. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Bate, M., Rushton, E. and Currie, D. A. (1991). Cells with persistent *twist* expression are the embryonic precursors of adult muscles in *Drosophila*. *Development* **113**, 79-89.

Bate, M., Rushton, E. and Frasch, M. (1993). A dual requirement for neurogenic genes in *Drosophila* myogenesis. *Dev. Suppl.* **1993**, 149-161.

Baylies, M. K. and Bate, M. (1996). *twist*: a myogenic switch in *Drosophila*. *Science* **272**, 1481-1484.

Baylies, M. K., Bate, M. and Ruiz Gomez, M. (1998). Myogenesis: a view from *Drosophila*. *Cell* **93**, 921-927.

Beiman, M., Shilo, B.-Z. and Volk, T. (1996). Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev.* **10**, 2993-3002.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-729.

Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation of *Drosophila*. *Development* **110**, 661-669.

Bopp, D., Burri, M., Baumgartner, S., Frigerio, G. and Noll, M. (1986). Conservation of a large protein domain in the segmentation gene *paired* and in functionally related genes of *Drosophila*. *Cell* **47**, 1033-1040.

Bopp, D., Jamet, E., Baumgartner, S., Burri, M. and Noll, M. (1989). Isolation of two tissue-specific *Drosophila* paired box genes, *Pox meso* and *Pox neuro*. *EMBO J.* **8**, 3447-3457.

Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M. and Nguyen, H. T. (1995). *Drosophila* MEF2, a transcription factor that is essential for myogenesis. *Genes Dev.* **9**, 730-741.

Bour, B. A., Chakravarti, M., West, J. M. and Abmayr, S. M. (2000). *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev.* **14**, 1498-1511.

Bourgouin, C., Lundgren, S. E. and Thomas, J. B. (1992). *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. *Neuron* **9**, 549-561.

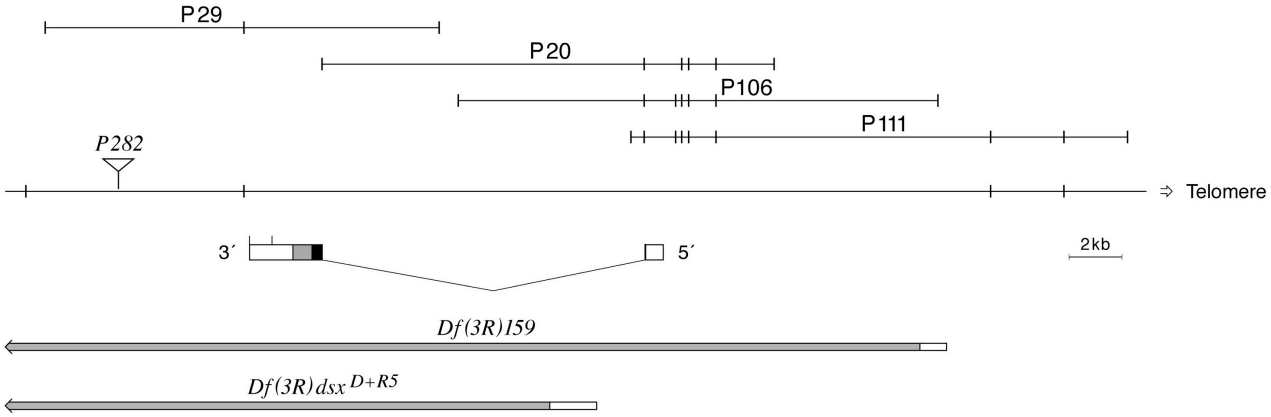
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brennan, K., Baylies, M. and Martinez Arias, A. (1999). Repression by Notch is required before Wingless signalling during muscle progenitor cell development in *Drosophila*. *Curr. Biol.* **9**, 707-710.

Buff, E., Carmena, A., Gisselbrecht, S., Jiménez, F. and Michelson, A. M. (1998). Signalling by the *Drosophila* epidermal growth factor receptor is required

for the specification and diversification of embryonic muscle progenitors. *Development* **125**, 2075-2086.
Campuzano, S. and Modolell, J. (1992). Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. *Trends Genet.* **8**, 202-208.
Carmena, A., Bate, M. and Jiménez, F. (1995). *lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. *Genes Dev.* **9**, 2373-2383.
Carmena, A., Gisselbrecht, S., Harrison, J., Jiménez, F. and Michelson, A. M. (1998a). Combinatorial signaling codes for the progressive determination of cell fates in the *Drosophila* embryonic mesoderm. *Genes Dev.* **12**, 3910-3922.
Carmena, A., Murugasu-Oei, B., Menon, D., Jiménez, F. and Chia, W. (1998b). *inscuteable* and *numb* mediate asymmetric muscle progenitor cell divisions during *Drosophila* myogenesis. *Genes Dev.* **12**, 304-315.
Carmena, A., Buff, E., Halfon, M. S., Gisselbrecht, S., Jiménez, F., Baylies, M. K. and Michelson, A. M. (2002). Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the *Drosophila* embryonic mesoderm. *Dev. Biol.* **244**, 226-242.
Castanon, I., Von Stetina, S., Kass, J. and Baylies, M. K. (2001). Dimerization partners determine the activity of the Twist bHLH protein during *Drosophila* mesoderm development. *Development* **128**, 3145-3159.
Chen, E. H., Pryce, B. A., Tzeng, J. A., Gonzalez, G. A. and Olson, E. N. (2003). Control of myoblast fusion by a guanine nucleotide exchange factor, Loner, and its effector ARF6. *Cell* **114**, 751-762.
Clark, I. B. N., Boyd, J., Hamilton, G., Finnegan, D. J. and Jarman, A. P. (2006). *D*-six4 plays a key role in patterning cell identities deriving from the *Drosophila* mesoderm. *Dev. Biol.* **294**, 220-231.
Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the *Drosophila* neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.
Crossley, A. C. (1978). The morphology and development of the *Drosophila* muscular system. In *The Genetics and Biology of Drosophila*. Vol. 2b (ed. M. Ashburner and T. R. F. Wright), pp. 499-560. New York: Academic Press.
Deák, P., Omar, M. M., Saunders, R. D. C., Pál, M., Komonyi, O., Szidonya, J., Maróy, P., Zhang, Y., Ashburner, M., Benos, P. et al. (1997). *P*-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. *Genetics* **147**, 1697-1722.
Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-2111.
Duan, H., Skeath, J. B. and Nguyen, H. T. (2001). *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. *Development* **128**, 4489-4500.
Duncan, I. W. and Kaufman, T. C. (1975). Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*: mapping of the proximal portion of the right arm. *Genetics* **80**, 733-752.
Dunin Borkowski, O. M., Brown, N. H. and Bate, M. (1995). Anterior-posterior subdivision and the diversification of the mesoderm in *Drosophila*. *Development* **121**, 4183-4193.
Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature* **374**, 464-467.
Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the *even-skipped* protein of *Drosophila*. *EMBO J.* **6**, 749-759.
Freeman, M. (1997). Cell determination strategies in the *Drosophila* eye. *Development* **124**, 261-270.
Frigerio, G., Burri, M., Bopp, D., Baumgartner, S. and Noll, M. (1986). Structure of the segmentation gene *paired* and the *Drosophila* PRD gene set as part of a gene network. *Cell* **47**, 735-746.
Fu, W. and Noll, M. (1997). The *Pax2* homolog *sparkling* is required for development of cone and pigment cells in the *Drosophila* eye. *Genes Dev.* **11**, 2066-2078.
Gisselbrecht, S., Skeath, J. B., Doe, C. Q. and Michelson, A. M. (1996). *heartless* encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Dev.* **10**, 3003-3017.
Gutjahr, T., Frei, E. and Noll, M. (1993a). Complex regulation of early *paired* expression: initial activation by gap genes and pattern modulation by pair-rule genes. *Development* **117**, 609-623.
Gutjahr, T., Patel, N. H., Li, X., Goodman, C. S. and Noll, M. (1993b). Analysis of the *gooseberry* locus in *Drosophila* embryos: *gooseberry* determines the cuticular pattern and activates *gooseberry neuro*. *Development* **118**, 21-31.
Gutjahr, T., Vanario-Alonso, C. E., Pick, L. and Noll, M. (1994). Multiple regulatory elements direct the complex expression pattern of the *Drosophila* segmentation gene *paired*. *Mech. Dev.* **48**, 119-128.
Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jiménez, F., Baylies, M. K. and Michelson, A. M. (2000). Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. *Cell* **103**, 63-74.
Jagla, T., Bellard, F., Lutz, Y., Dretzen, G., Bellard, M. and Jagla, K. (1998). *ladybird* determines cell fate decisions during diversification of *Drosophila* somatic muscles. *Development* **125**, 3699-3708.
Jagla, T., Bidet, Y., Da Ponte, J. P., Dastugue, B. and Jagla, K. (2002). Cross-repressive interactions of identity genes are essential for proper specification of cardiac and muscular fates in *Drosophila*. *Development* **129**, 1037-1047.
Jan, Y. N. and Jan, L. Y. (1993). HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* **75**, 827-830.
Kiehart, D. P. and Feghali, R. (1986). Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol.* **103**, 1517-1525.
Klemenz, R., Weber, U. and Gehring, W. J. (1987). The *white* gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucleic Acids Res.* **15**, 3947-3959.
Knirr, S., Azpiazu, N. and Frasch, M. (1999). The role of the NK-homeobox gene *slouch* (*S59*) in somatic muscle patterning. *Development* **126**, 4525-4535.
Lee, H.-H. and Frasch, M. (2000). Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target *sloppy paired*. *Development* **127**, 5497-5508.
Michelson, A. M. (1994). Muscle pattern diversification in *Drosophila* is determined by the autonomous function of homeotic genes in the embryonic mesoderm. *Development* **120**, 755-768.
Michelson, A. M., Abmayr, S. M., Bate, M., Martinez Arias, A. and Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes Dev.* **4**, 2086-2097.
Michelson, A. M., Gisselbrecht, S., Zhou, Y., Baek, K.-H. and Buff, E. M. (1998). Dual functions of the Heartless fibroblast growth factor receptor in development of the *Drosophila* embryonic mesoderm. *Dev. Genet.* **22**, 212-229.
Noll, M. (1993). Evolution and role of *Pax* genes. *Curr. Opin. Genet. Dev.* **3**, 595-605.
Nose, A., Isshiki, T. and Takeichi, M. (1998). Regional specification of muscle progenitors in *Drosophila*: the role of the *msh* homeobox gene. *Development* **125**, 215-223.
Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R. and Leptin, M. (1997). Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development* **124**, 2915-2922.
Riechmann, V., Rehorn, K.-P., Reuter, R. and Leptin, M. (1998). The genetic control of the distinction between fat body and gonadal mesoderm in *Drosophila*. *Development* **125**, 713-723.
Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D. M., Benz, W. K. and Engels, W. R. (1988). A stable genomic source of *P* element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.
Rodríguez, I., Hernández, R., Modolell, J. and Ruiz-Gómez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* **9**, 3583-3592.
Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-1202.
Ruiz Gómez, M. and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires Numb. *Development* **124**, 4857-4866.
Ruiz-Gómez, M., Romani, S., Hartmann, C., Jäckle, H. and Bate, M. (1997). Specific muscle identities are regulated by *Krüppel* during *Drosophila* embryogenesis. *Development* **124**, 3407-3414.
Ruiz-Gómez, M., Coutts, N., Price, A., Taylor, M. V. and Bate, M. (2000). *Drosophila* Dumbfounded: a myoblast attractant essential for fusion. *Cell* **102**, 189-198.
Ruiz-Gómez, M., Coutts, N., Suster, M. L., Landgraf, M. and Bate, M. (2002). *myoblasts incompetent* encodes a zinc finger transcription factor required to specify fusion-competent myoblasts in *Drosophila*. *Development* **129**, 133-141.
Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene, *myoblast city,* provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* **121**, 1979-1988.
Sink, H., Rehm, E. J., Richstone, L., Bulls, Y. M. and Goodman, C. S. (2001). *sidestep* encodes a target-derived attractant essential for motor axon guidance in *Drosophila*. *Cell* **105**, 57-67.
Staehling-Hampton, K., Hoffmann, F. M., Baylies, M. K., Rushton, E. and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila. Nature* **372**, 783-786.
Stathopoulos, A., Tam, B., Ronshaugen, M., Frasch, M. and Levine, M. (2004). pyramus and thisbe: FGF genes that pattern the mesoderm of *Drosophila* embryos. *Genes Dev.* **18**, 687-699.
Tapanes-Castillo, A. and Baylies, M. K. (2004). Notch signaling patterns *Drosophila* mesodermal segments by regulating the bHLH transcription factor *twist*. *Development* **131**, 2359-2372.
Van Vactor, D., Sink, H., Fambrough, D., Tsoo, R. and Goodman, C. S. (1993). Genes that control neuromuscular specificity in *Drosophila*. *Cell* **73**, 1137-1153.
Yin, Z. and Frasch, M. (1998). Regulation and function of *tinman* during dorsal mesoderm induction and heart specification in *Drosophila*. *Dev. Genet.* **22**, 187-200.

A



B

stop (*Poxm*^{R361})

```
  1 MDPESQCPQYGEVNQLGGVFVNGRPLPNATRMRIVELARLGIRPCDISRQLRVSHGCVSKILARYHETGS
 71 ILPGAIGGSKPRVTTPKVVNYIRELKQRDPGIFAWEIRDRLLSEGICDKTNVPSVSSISRILRNKLGSLG
141 HQHTPGTVMGSGSSSGGGSVSSNGGQNNGTSASNNINLSNLGNPGGGPHHPHHHHHHQSAAAAASAHHVH
211 AHAHAHAHLYNSIYQPYSAAAAYSMKTPCGSPSPPQGAGGQGSVPHPHQLRSVAAAAAAAHWPSSHSVSD
281 ILAHHQAVALRASCQVGVGVGGMGGMGSTVSPLPMTPSPVAGTAGGQPLLDCEGGAGQQSPYNYYMYFQN
351 GGMHHHHHHGGMMAAGATGL 370
```

Fig. S1. Structural organization of the *Poxm* gene and Poxm protein sequence. (**A**) Overlapping DNA fragments, covering the *Poxm* locus, P29, P20, P106 and P111, and isolated from a genomic library in λ phage EMBL4 (Bopp et al., 1989), are shown at the top with respect to an *Eco*RI restriction map below, in which the location of the P-element insertion P282 (Deák et al., 1997) is indicated. The locations and orientations of the two Poxm exons (Bopp et al., 1989), shown below the restriction map, were derived by sequencing of two Poxm cDNAs, P29c1 and P29c2, differing mainly in the lengths of their trailers, and of the corresponding genomic DNA, whereas the transcriptional start site was determined by 5' RACE and coincides with the 5' end of P29c1 (the GenBank accession number for the full-length Poxm cDNA P29c1, is DQ459353; for P29c2 it is DQ459354). The exons corresponding to the full-length Poxm transcript indicate the open reading frame (shaded) with the paired-domain (black), the untranslated leader and trailer (white), and two different poly(A) addition sites found in two cDNAs (vertical marks). Two deficiencies, *Df(3R)159* and *Df(3R)dsx*$^{D+R5}$, are indicated at the bottom. Whereas their proximal breakpoints map proximal to the genomic region shown, their distal breakpoints are located within the regions delimited by the open boxes. (**B**) Amino acid sequence of Poxm derived from the longest open reading frame of Poxm cDNAs. The previously published paired-domain and octapeptide (Bopp et al., 1989; Noll, 1993) are underlined, and the location of the EMS-induced amber mutation *Poxm*^{361}, Q15stop, is indicated by an arrow. The black triangle marks the position of the intron. Note that amino acids 5-370 are identical to 37-402 of the Poxm protein published by FlyBase, whereas our N-terminal amino acids deviate because the first exon has been incorrectly predicted by FlyBase.

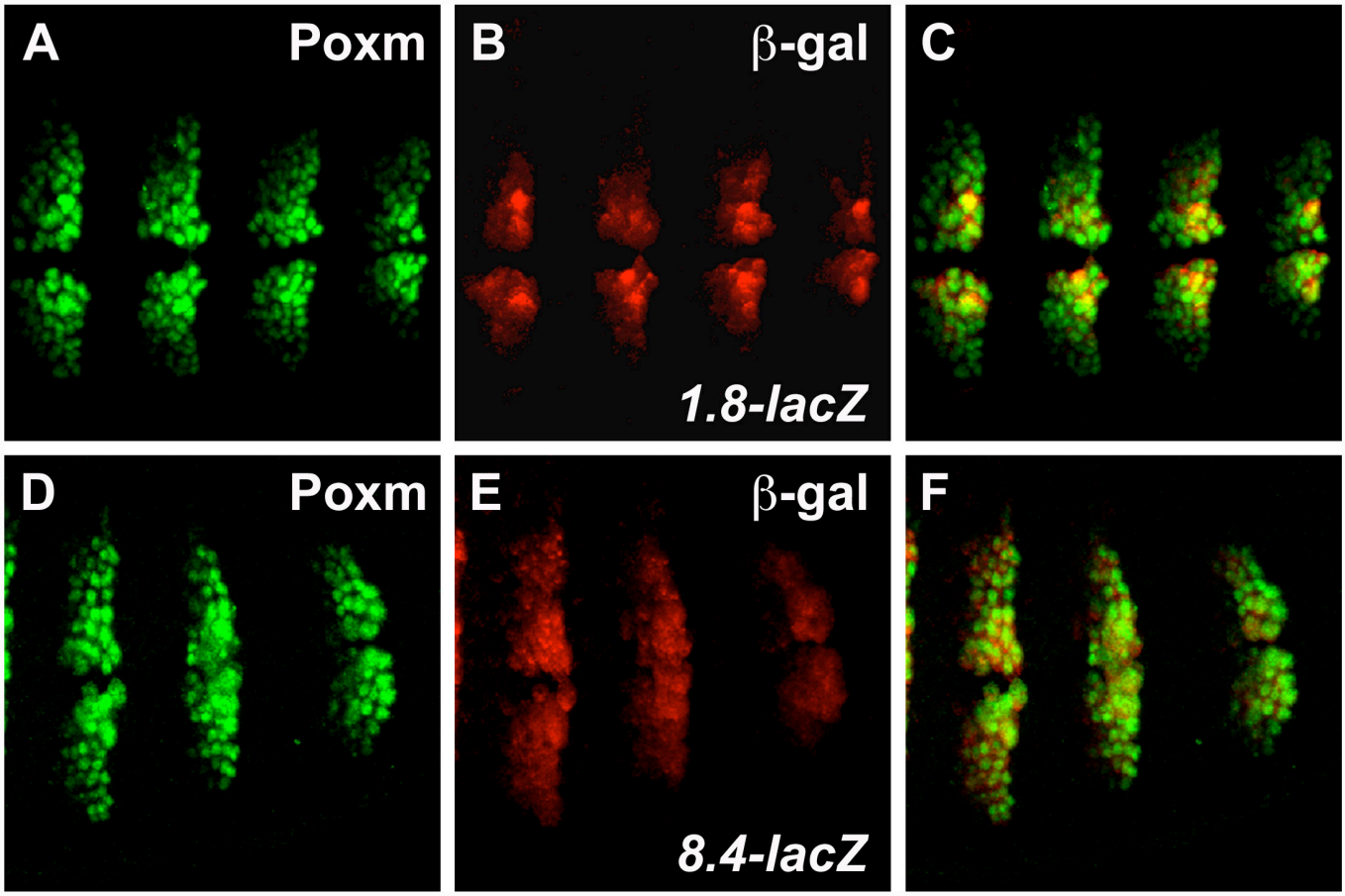


Fig. S2. ***lacZ*** **expressed under the direct control of the** ***Poxm*** **enhancers coincides with endogenous Poxm protein.** Transgenic embryos at early stage 11, expressing lacZ under the direct control of the 1.8 kb fragment (**A-C**) or the 8.4 kb fragment (**D-F**), both including the early enhancer of Poxm (Fig. 3E), were stained for Poxm (A,D) and β-gal (B,E). The two expression patterns completely coincide, and no ectopic β-gal is observed (C,F). Note that Poxm is nuclear whereas β-gal is cytoplasmic.

Poxm	
muscle 11	muscle 18
N	N
A	A
N	A
D	M
N	N
N	N
N	A
N	M
N	A
A	N
N	N
N	N
N	M
A	M
A	N
A	N
A	A
N	N
A	N
D	M
D	M
D	M
A	A
N	A
D	M
A	A
D	M
D	M
D	A
D	N
D	M
D	M
A	N
N	A
D	D
N	N
N	A
D	A
A	N
D	A
A	N
A	N
N	A
A	A
A	N
A	A
D	N
A	N
N	A
A	M
D	M
A	M

l(1)sc; Poxm	
muscle 11	muscle 18
A	M
D	M
D	M
D	M
A	M
A	M
A	A
N	N
D	A
A	A
D	A
N	A
A	A
A	M
A	A
N	M
N	M
A	A
N	M
D	M
N	A
N	A
D	M
A	A
N	A
D	M
D	M
A	M
A	A
D	M
N	M
D	M
A	M
D	M
D	M
N	M
D	M
D	M
A	N
D	M
D	M
D	M
D	M
D	M
D	M
D	M
N	A
D	M
N	A
D	M

Poxm	
muscle 11	muscle 18
N	N
N	N
N	N
D	M
D	M
N	A
N	N
N	N
N	N
N	N
D	M
D	M
D	M
D	M
D	N
N	N
D	M
N	N
N	N
N	N
A	A
N	N
D	M
N	A
N	A
N	M
D	A
N	N
D	M
A	N
N	N
A	N
N	N
N	N
D	M
N	A
A	M
A	M
N	N
N	N
D	A
A	M
D	M
A	M
A	M
D	N
D	M
N	M
A	M
D	M
A	A

l(1)sc; Poxm	
muscle 11	muscle 18
N	M
N	M
N	M
N	A
D	A
N	M
A	M
A	A
M	M
M	M
A	A
D	M
A	M
M	M
M	M
M	M
D	M
D	M
A	M
D	M
D	M
D	N
D	M
A	M
N	M
D	M
A	M
D	M
D	A
D	M
A	A
D	N
A	M
A	M
D	M
D	M
D	A
D	M
A	M
D	N
D	M
A	M
D	M
D	M
D	M
N	N
D	M
A	M
D	M
N	M
A	M
D	N
A	A
A	M
A	M
D	M

l(1)sc; Poxm	
muscle 11	muscle 18
A	M
A	M
D	M
D	M
D	M
A	M
D	M
N	N
D	M
D	M
D	M
A	M
D	M
D	M
D	A
A	M
A	M
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N	M
A	M
D	M
D	A
D	A
D	M
A	A
D	A
D	M
N	N
D	A
D	N
D	M
D	M
D	M
D	M

Table S1. Phenotypes of muscles DO3 and DT1 in individual hemisegments of Poxm and *l(1)sc*; *Poxm* mutants. Mutant phenotypes, analyzed as described in the legend to Fig. 5, are given for muscles DO3 and DT1 of individual hemisegments, as summarized in Fig. 5A,D. Each line lists the phenotypes of a hemisegment for muscles DO3 and DT1 of $Poxm^{R361}$ mutants (left column, 108 hemisegments) and of $Df(1)sc^{19}/Df(1)sc^{19}$ or *Y*; $Poxm^{R361}$ mutants (right column, 168 hemisegments). Muscle phenotypes were classified as missing (M, red), abnormal (A, yellow), duplicated (D, purple) and normal wild type (N, green).

Chapter 3

Roles of the *Pax3/7* Homolog *gsb* in *Drosophila* Myogenesis and Its Genetic Interactions with *Poxm* and *D-Six4*

Summary

Pax 3 and *Pax 7* play essential roles in vertebrate myogenesis. *gsb* is the only *Pax3/7* homolog in *Drosophila* expressed in the mesoderm. However, its developmental functions in the mesoderm remain unknown. Detailed analyses of the mesodermal expression patterns of Gsb, the muscle phenotype of *gsb* mutants, and the effects of overexpressing Gsb throughout the mesoderm showed that *gsb* is necessary for the proper development of *Drosophila* somatic body wall muscles, especially those derived from the ventral region of the mesoderm. In addition, genetic analyses show that *gsb* regulates another Pax gene, *Poxm*, but is not regulated by *Poxm* or the Six family homeodomain protein D-Six4. Our results led us to characterize the "Pax-Six gene network" in *Drosophila* myogenesis.

Introduction

A key question in developmental biology is how specific cell fates are established by temporal and spacial cues through precise genetic regulations. The specification of the mesoderm in *Drosophila melanogaster* provides an excellent paradigm to study how cells that are initially identical can acquire different fates by expressing specific sets of cell-type regulators. Several organs can arise from the *Drosophila* mesoderm, including the muscles of the gut (visceral muscles), the body wall muscles (somatic muscles), the heart, the fat body, and the gonads (Bate, 1993). Thus, the selection of cells undergoing myogenesis is part of a more general process in which the progenitors of different mesodermal tissues are formed.

Each of the abdominal hemisegments A2-A7 has 30 identifiable individual muscles (Bate, 1993) that develop from the somatic mesoderm. This is initiated when the invaginated mesoderm migrates dorsolaterally under the ectoderm (Beiman et al., 1996; Gisselbrecht et al., 1996). During this process, the ectodermal Wingless (Wg) signal maintains the activity of the segmentation gene *sloppy paired* (*slp*) (Lee and Frasch, 2000; Riechmann et al., 1997), which restricts high levels of the bHLH transcription factor Twist (Twi) to the mesodermal regions below the posterior portions of the ectodermal parasegments (Baylies et al., 1998). These high levels of Twi function as myogenic switch, separating the posterior somatic and cardiac mesoderm from the anterior visceral mesoderm and fat body (Baylies and Bate, 1996; Dunin Borkowski et al., 1995). When the dorsal migration of the mesoderm is complete, these metamerically repeated high Twi domains are further subdivided along the dorsoventral axis by the ectodermal signal Dpp (Staehling-Hampton et al., 1994). This signal restricts transcription of *tinman* (*tin*) to the dorsal mesoderm, where its homeodomain protein specifies heart and dorsal somatic mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993; Bodmer et al., 1990; Frasch, 1995). On the other hand, determinants of the non-dorsal somatic mesoderm are just beginning to be characterized. In Chapter 2, I have shown that *Poxm* is crucial for the proper development of many ventral and lateral muscles. However, because the penetrance of the muscle phenotype in *Poxm* mutants is still relatively low, other genes may also be involved in the somatic myogenesis of *Drosophila*.

One of the candidates is *gsb*, a member of the *Pax3/7* subfamily. In vertebrate myogenesis, *Pax3* and *Pax7* play essential roles (reviewed by Buckingham and Relaix, 2007). In *Drosophila*, although it has been reported that Gsb is expressed in the mesoderm (Gutjahr et al., 1993), a time course study of this expression pattern and the functions of *gsb* in myogenesis remain to be analyzed. In addition, it will be interesting to know the regulatory relationships among *gsb*, *Poxm*, and other determinants of the somatic mesoderm, so that the *Drosophila* myogenic program can be understood.

Here, I show that during early embryonic stages, *gsb* is expressed in the somatic mesoderm but not in the heart primordium. It is necessary for the proper development of *Drosophila* somatic body wall muscles, especially those derived from the ventral region of the mesoderm. Genetic analyses show that *gsb* regulates another Pax gene, *Poxm*, but is not regulated by Poxm or the Six family homeodomain protein D-Six4.

Materials and Methods

Immunohistochemistry and microscopy

The following primary antisera were used: rabbit anti-Poxm (Duan et al., 2007), rat anti-Gsb (Zhang et al., 1994), rabbit anti-MHC (myosin heavy chain; Kiehart and Feghali, 1986), rabbit anti-Twist (Roth et al., 1989), rabbit anti-b-galactosidase (Cappel), rabbit anti-Tin (Yin and Frasch, 1998), rabbit anti-Kr (kindly provided by Herbert Jaeckle), mouse anti-Connectin (Meadows et al., 1994), and rabbit anti-GFP (Medical & Biological Laboratories). Embryos were fixed and stained as described previously (Gutjahr et al., 1993).

Muscle patterns were visualized after staining with anti-MHC. The fluorescent signals were amplified by tyramide signal amplification (TSA; kits #12 and #25 from Invitrogen), and embryos were analyzed with a Leica SP1 confocal microscope.

Fly stocks.

The following fly stocks were used:

y w,

w^{1118}; $Poxm^{R361}$ *red/TM3*, *Sb Ser P{w+; hb-lacZ}* (Duan et al., 2007),

y w; gsb^{s252}*/SM6B, eve-lacZ* (He, 2007),

y w; gsb^{J46}*/CyO, Kr-GFP* (He, 2007),

D-Six4^{289}*/ TM3* (Kirby et al., 2001),

twi-Gal4 (Bloomington stock 914),

w; *UAS-Gsb-7* (Jiao et al., 2001).

Results

Expression of *gsb* in the somatic mesoderm during myogenesis

It was shown earlier that the Gsb protein appears in a characteristic segment-polarity pattern of stripes at gastrulation and persists until head involution. It is initially restricted to the ectodermal and neuroectodermal germ layer, but is later detected in mesodermal and neuronal cells as well (Gutjahr et al., 1993). However, the expression of Gsb in the mesoderm has never been characterized in detail, partially because of lack of appropriate mesodermal cell markers. The use of antibodies

against Twi, Poxm, and Gsb to perform multiple immunofluorescence labeling permits one to study the expression of Gsb in the developing mesoderm. The following analysis is focusing on the abdominal segments. In stage 9 embryos, when the segmental fluctuation of Twi expression along the A/P axis is not yet pronounced (Fig. 1A), Gsb is expressed in a segmentally reiterated pattern of stripes in the ectoderm (Fig. 1B), while its expression in the somatic mesoderm is hardly detectable since few, if any, cells coexpress Twi and Gsb (Fig. 1C). During stage 10, it becomes clear that a group of cells located in the vetral-lateral region of each segment coexpress Gsb and Twi (Fig. 1D-F). These cells belong to the somatic mesoderm, as they are located in the high Twi domain and colocalize with Poxm expressing cells (Fig. 1G-I). During germ band retraction, Gsb disappears from most of the mesodermal cells, and by stage 12 it is only maintained in the most ventral Poxm-expressing cell, which is either a muscle founder cell or the ventral adult muscle precursor cell (Fig. 1J-L). However, at stage 15, Gsb cannot be detected in the muscle fibers (Fig. 1M-O).

To further identify the nature of the cells epressing Gsb in the mesoderm, I stained wild-type stage 11 embryos with antibodies against Gsb and Tin (Fig. 2). Cells expressing Gsb are adjacent and ventral to, but do not overlap with, the cells in the dorsal region where Tin is expressed stongly. Therefore, these Gsb expressing cells will not form part of the heart primordium.

Somatic muscle patterns are severely disrupted in *gsb* mutant embryos

The expression of Gsb in the somatic mesoderm suggests that it plays a role in myogenesis. To specify its functions during the formation of *Drosophila* larval body wall muscles, the muscle phenotype of two *gsb* alleles were analyzed. These were generated by Haihuai He in our lab, gsb^{J46} through imprecise P-element excision and gsb^{s252} by homologous recombination (He, 2007). Muscle patterns were visualized after anti-MHC staining. Compared to wild-type embryos (Fig. 3A, B), both gsb^{J46} and gsb^{s252} embryos show severely disturbed muscle patterns with the muscle phenotype of gsb^{s252} embryos being stronger (Fig. 3C-F). This is consistent with the idea that gsb^{s252} is a null and gsb^{J46} a hypomorphic allele (He, 2007). Therefore, the following analyses focused only on the muscle phenotype of gsb^{s252} embryos. Notably, the ventral muscles VO1-6 and VA1-3 are frequently missing, while VT1 and VL1-4 are often abnormal in gsb^{s252} embryos. The lateral and dorsal muscles

appear unaffected, with the occasional exception of an abnormal lateral muscle LL1-4. These observations show that the functions of *gsb* are most important for the proper development of the ventral muscles.

The expression of *Poxm* is down-regulated in *gsb* mutants

As shown above, in gsb^{s252} embryos, the ventral muscles are affected most strongly, which is also true for *Poxm* mutants. Thus, it is possible that *gsb* performs its functions, at least partially, by regulating another Pax gene, *Poxm.* Indeed, Poxm expression is affected in gsb^{s252} embryos throughout embryogenesis. At late stage 10, instead of forming the triangular shape typical for Poxm expression during early stages (Fig. 4A), Poxm protein was only detected in the most ventral region close to the ventral midline and disappeared from the lateral regions of the mesoderm (Fig. 4B). In the abdominal segments of stage 12 wild-type embryos, Poxm is restricted to the ventral adult muscle precursor (VaP) and founder cells of muscles DO3, DT1, and VA1-3 (Fig. 4C). In *gsb* mutants, however, while it is still expressed in the ventral region of the thoracic segments (indicated by asterisks in Fig. 4D), Poxm is maintained only in the founders of the lateral muscles DO3 and DT1 and disappears from the ventral region in the abdominal segments (Fig. 4D). At stage 15, after the formation of muscle fibers, Poxm is still expressed in muscles DT1 and VA1-3 of wild-type embryos (Fig. 4E), whereas in gsb^{s252} embryos it is absent from the ventral muscles VA1-3 and expressed only in muscle DT1. These results are consistent with the muscle phenotype of gsb^{s252} mutants observed after staining with anti-MHC (Fig. 3C, D).

The expression of muscle identity genes *Kr* and *Connectin* is also affected in *gsb* mutants

Since the effects on late Poxm expression of losing functional *gsb* may exclusively result from *Poxm* being a target of *gsb*, I examined the expression of other muscle identity genes in gsb^{s252} embryos to see if the affected muscles lose their identities completely. Unlike in wild-type embryos at stage 15, where Kr is expressed in three groups of muscles located in the dorsal, lateral, and ventral regions (Fig. 5B), Kr expression in gsb^{s252} embryos only appears normal in the dorsal and lateral groups while it is lost in many ventral muscles (indicated by asterisks in Fig. 5A). The same is true for the expression of another muscle identity gene, Connectin. In stage 15

wild-type embryos, Connectin expression can be detected in the lateral muscles DT1 and LT2-4 as well as in the ventral muscles VA1 and VA2 (Fig. 5D). However, in gsb^{s252} embryos, Connectin is not expressed in the ventral muscles while its expression in the lateral region remains largely unaffected (Fig. 5C). These results indicate that in the absence of functional Gsb, many of the ventral muscles cannot express the proper set of muscle identity genes and hence lose their identities.

Gsb expression in the mesoderm is not regulated by *Poxm* and *D-Six4*

Since *Poxm* is activated by *gsb* and the cells expressing Gsb in the mesoderm are within the "Poxm expressing domain" (Fig. 1G-I), it would be interesting to see whether *Poxm* regulates the mesodermal expression of Gsb as well and therefore the two form a feedback loop. However, double staining for Twi and Gsb shows that mesodermal Gsb expression is unaffected in $Poxm^{R361}$ embryos (Fig. 6D-F) as compared to wild-type embryos (Fig. 6A-C).

In vertebrate myogenesis, it has been shown that Six1 and Six4 homeoproteins are required for Pax3 expression (Grifone et al., 2005). In *Drosophila*, the *Six4/5* family member *D-Six4* has been reported to be required for the development of specific muscles that arise from the dorsolateral and ventral regions (Clark et al., 2006). Since *gsb* belongs to the *Pax3/7* subfamily, one might suspect that *gsb* is similarly activated by *D-Six4*. However, the expression pattern of Gsb in the mesoderm of $D\text{-}Six4^{289}$ mutants (Fig. 6G-I) does not differ from that of wild-type embryos (Fig. 6A-C).

There are no obvious correlations between cells expressing both Poxm and Gsb and those where Poxm expression is abolished in *gsb* mutants

Since the early expression of *Poxm* is down-regulated in *gsb* mutants, it will be interesting to know whether this is a cell-autonomous effect. If so, one would expect that in and only in the mesodermal cells that express Gsb, Poxm expression is affected when Gsb is absent. To follow the fate of Gsb expressing cells in *gsb* mutants, the hypomorphic allele gsb^{J46} was used, which had been generated by imprecise excision of *P{P1155}*, a P-element insertion located 54 bp upstream of the *gsb* transcription start site (Duman-Scheel et al., 1997; Liu, 2003; He, 2007). After excision, gsb^{J46} retains 2,147 bp of the *lacZ* coding region and is expected to produce a protein containing the N-terminal half of β-Galactosidase whose expression is controlled by

the endogenous *gsb* enhancers (He, 2007). Staining gsb^{J46}/*CyO, Kr-GFP* embryos with anti-β-Gal and anti-Gsb simultaneously shows that the expression of β-Gal is almost identical to that of endogenous Gsb (Fig. 7A-C). Therefore, in gsb^{J46} mutants, one can identify cells that would express Gsb in a wild-type situation by anti-β-Gal staining. Double-staining of Poxm and β-Gal in gsb^{J46}/ gsb^{s252} embryos shows that some of the β-Gal expressing cells also express Poxm, while others only express β-Gal (Fig. 7D-F). However, the number of cells in which Poxm expression is abolished in *gsb* mutants (Fig. 4A, B) exceeds the number of cells that express only β-Gal (Fig. 7D-F). These results demonstrate that the expression of Poxm is abolished in cells of *gsb* mutants that would not express mesodermal Gsb in the wild type. On the other hand, cells that express both Poxm and Gsb do not necessarily fail to express Poxm upon removal of functional Gsb (Fig. 7D-F).

The early expression of Poxm is not affected when Gsb is ubiquitously expressed in the mesoderm

To test whether ubiquitous expression of *gsb* in the entire mesoderm can lead to ectopic Poxm expression, *UAS-gsb* was expressed under the control of *twi-Gal4*, which is active throughout the mesoderm preceding muscle differentiation (Thisse et al., 1988; Bate et al., 1991). In these embryos, the expression of Poxm at early and late stage 11 appears to be indistinguishable from wild-type embryos (Fig. 8). Therefore, although *gsb* is necessary for the activation of Poxm during early embryogenesis, ectopic expression of *gsb* alone is not sufficient to induce ectopic expression of Poxm.

Late expression of Poxm in muscle founders and differentiated muscle fibers is affected when Gsb is ectopically expressed in the mesoderm

Since expression of Poxm during early and late embryonic stages is regulated differently (Duan et al., 2007), I also examined Poxm expression in muscle founders and differentiated muscle fibers during late stages in embryos expressing *twi-Gal4/UAS-gsb*. Interestingly, in these embryos, the expression of Poxm in the ventral muscles is similar to that in wild-type embryos (Fig. 9). However, at both late stage 12 and stage 15, the expression of Poxm in muscle DT1 disappears in some segments when *gsb* is ectopically expressed (indicated by asterisks in Fig. 9B, D).

The pattern of somatic body wall muscles is affected when *gsb* is ectopically expressed in the mesoderm

The effect of ectopic Gsb on late Poxm expression can be an indirect effect caused by the disruption of the overall muscle pattern. To test this possibility, stage 16 embryos expressing *twi-Gal4/UAS-gsb* were stained with anti-MHC. As compared to wild-type embryos, these embryos show a disturbed pattern of their somatic body wall muscles (Fig. 10). While the ventral and dorsal muscles are largely normal, the dorsolateral muscles DT1, DO3, and DO4 are frequently abnormal (indicated by asterisks in Fig. 10B). These observations show that although the muscle phenotype of *gsb* mutants is very severe, when misexpressed only in the mesoderm, *gsb* can only affect the development of a few dorsolateral muscles, without causing the maldevelopment of the majority of the muscles.

Discussion

I have shown in this chapter that during early embryonic stages, *gsb* is expressed in the somatic mesoderm but not in the heart primordium. It is necessary for the proper development of *Drosophila* somatic body wall muscles, especially muscles derived from the ventral region of the mesoderm. However, when misexpressed in the mesoderm, *gsb* cannot cause severe disruption of the muscle pattern. *gsb* performs its functions partially through the regulation of *Poxm*, which is another key player in *Drosophila* embryonic myogenesis. Although cells expressing Gsb in the mesoderm are located within the "Poxm expressing domain", mesodermal Gsb expression is not regulated by Poxm. In addition, unlike in vertebrates where Six1 and Six4 homeoproteins are required for Pax3 expression, mesodermal expression of the *Pax3/7* family member *gsb* is not regulated by the Six family homeodomain protein D-Six4. These results help us to understand the complex regulatory network of *Drosophila* myogenesis. By comparing this process with that in vertebrates, one can further gain new insights into how the gene networks for muscle development have evolved and how a muscle develops in vertebrates.

The *gsb* gene is necessary for proper muscle development but is not sufficient to disrupt the muscle patterning when misexpressed in the mesoderm

Almost all ventral muscles disappear or appear abnormal in gsb^{s252} embryos. This is

confirmed by analyzing the expression of a few muscle identity genes, *Poxm*, *Kr*, and *Connectin*, which is lost in the ventral muscles of stage 15 *gsb* mutant embryos, while it remains largely unaffected in the dorsal and lateral regions. Furthermore, to determine the stage of development at which the muscles are affected, I have also examined the expression of Poxm during earlier stages and found no Poxm in the ventral muscle founders of gsb^{s252} embryos. Because *gsb* is expressed in only one of the "Poxm expressing cells" at stage 12 (Fig. 1J-L), the absence of Poxm in all ventral founders cannot be explained simply by *Poxm* being a target of *gsb*. It is more likely that those ventral muscle founders are not generated in *gsb* mutants. Thus, the requirement for *gsb* in muscle development occurs quite early during myogenesis, most probably before and during the specification of muscle founder cells. Because *gsb* is expressed not only in the mesoderm but also in the ectoderm (Gutjahr et al., 1993) and cell fates in the mesoderm can be affected by signals that are received from the overlying ectoderm (Bate and Baylies, 1996; Azpiazu et al., 1996; Tajbakhsh and Cossu, 1997), the question arises whether the muscle phenotype of *gsb* mutants originates from its absence in the mesoderm, the ectoderm, or both. The ideal way to address this question would be by providing Gsb only in the mesoderm of gsb^{s252} embryos to see whether the muscle phenotype can be rescued. Before doing that, the effect of overexpressing Gsb throughout the mesoderm in otherwise wild-type embryos was examined. Interestingly, in such embryos where pan-mesodermal expression of Gsb was promoted under the control of *twi-Gal4*, most of the somatic body wall muscles appeared unaffected, except the dorsolateral muscles DT1, DO3, and DO4 which were frequently abnormal. This result shows that the elevated level of Gsb protein alone cannot cause the maldevelopment of most muscles, except the 3 dorsolateral muscles DT1, DO3, and DO4 which, however, develop normally in the absence of *gsb*. In addition, because neither muscle duplication nor extra muscles were found when *gsb* was expressed throughout the mesoderm, cells of other mesodermal tissues may not lose their identities and enter myogenesis in the presence of ectopic Gsb.

Since Wg is required for muscle formation (Baylies et al., 1995) and its maintenance depends on *gsb* (Li and Noll, 1993), the phenotype observed in *gsb* mutants can be an indirect effect caused by the downregulation of Wg. Like Gsb, Wg is also expressed in both the ectoderm and the mesoderm, but exclusive expression of *wg* in the ecdoderm can rescue mesodermal defects in *wg* mutant embryos (Baylies et

al., 1995). Therefore, it will be interesting to see whether forced expression of Wg in the ectoderm of *gsb*^{s252} embryos can rescue the muscle phenotype of *gsb* mutants.

The Pax-Six gene network: which and how?

Genes from the Pax and Six families form regulatory networks that are involved in the development of many tissues and organs in many animal spieces (Kardon et al., 2004). However, which members of these gene families are involved and how they regulate each other vary with different developmental processes. A well studied case is the network containing the Pax genes *twin of eyeless* (*toy*) and *eyeless* (*ey*) and the Six gene *sine oculis* (*so*) employed in *Drosophila* eye development (Chen et al., 1997; Shen and Mardon 1997; Pignoni et al., 1997; Halder et al., 1998; Czerny et al., 1999). Vertebrate homologs of these genes have also been identified and a subset of them is involved in eye development. Indeed, *Pax6*, which is the pro-ortholog of *ey* and *toy*, is required for vertebrate eye formation (Hill et al., 1991). However, the *Six3/6* subfamily members *Six3* and *Optix2*, which are involved in vertebrate eye development (Gallardo et al., 1999; Wallis et al., 1999), are not orthologs of the *Six1/2* subfamily member *so*. Thus, it seems that during evolution, different members of the Pax and Six families have been substituted in the network. In addition, the regulatory relationships among components of the network from different gene families are also distinct in different organisms. For instance, in *Drosophila* eye development, *ey* activates *so* but is not regulated in turn by *so* (Chen et al., 1997; Halder et al., 1998). In vertebrate eye development, however, the expression of *Six3* does not depend on *Pax6* (Oliver et al., 1995). In general, it appears that although in both *Drosophila* and vertebrates, the regulatory network of eye development involves members of the Pax and Six families, the way they are deployed are different.

Are these differences unique to eye development, or do they also apply to other developmental processes? Comparing the similarities and differences of muscle development in *Drosophila* and vertebrates may provide new insights. Like vertebrates, *Drosophila* divides its mesoderm into segmental units from which the myogenic linage arises. Although many genes involved in the myogenic pathway are well conserved from flies to vertebrates, their specific functions seem to differ in different organisms (reviewed by Baylies and Michelson, 2001). In vertebrate myogenesis, *Pax3* and *Pax7* play essential roles (reviewed by Buckingham and Relaix, 2007), and the expression of *Pax3* is controlled by *Six1 and Six4* (Grifone et al., 2005).

In *Drosophila* embryos, the *Pax1/9* ortholog *Poxm* is required for the proper development of many ventral and lateral body wall muscles (Duan et al., 2007), and in this chapter I showed that the *Pax3/7* ortholog *gsb* is also required for the formation of many ventral muscles. In addition, *D-Six4*, the *Drosophila* ortholog of *Six4/5*, is expressed in the somatic mesoderm and is required for the development of specific muscles that arise from the dorsolateral and ventral regions (Clark et al., 2006). Thus, at least two genes from the Pax family and one gene from the Six family are involved in the embryonic myogenesis of *Drosophila*, and all of them are required for the proper development of ventral muscles. Considering the situation in vertebrate, it is interesting to know the regulatory relationships among them, namely which of them are involved in the "Pax-Six network" of *Drosophila* myogenesis and how they regulate each other. Surprisingly, unlike in vertebrates where the expression of *Pax3* depends on *Six1* and *Six4*, the mesodermal expression of the *Pax3/7* ortholog *gsb* is unaffected in *D-Six4* mutants. However, the expression of *Poxm*, a member of the *Pax1/9* subfamily, depends on *D-Six4* (Zhang and Noll, unpublished data). While mesodermal Gsb expression is not affected in *Poxm* mutants, the regulation of *Poxm* by *gsb* is more complicated, as *gsb* is expressed in both the mesoderm and the ectoderm, and mesodermal cell fates can be affected by the overlying ectoderm. It is clear that the expression of Poxm is markedly down-regulated in *gsb* mutants, but there seems to be no certain correlations between cells expressing both Poxm and mesodermal Gsb in wild-type embryos and those in which Poxm expression is abolished in *gsb* mutants. In *gsb* mutants, the down-regulation of Poxm cannot be simply explained by the absence of Gsb in these cells because some of these cells have never expressed Gsb and some of them, though expressing Gsb, still express Poxm in *gsb* mutants. Because Wg is required for the expression of *Poxm* (Chen, 2003) and its maintenance depends on *gsb* (Li and Noll, 1993), it is very likely that the down-regulation of *Poxm* observed in *gsb* mutants is caused partially by the down-regulation of Wg in the absence of ectodermal Gsb. To summarize, in the *"Pax-Six network"* of *Drosophila* myogenesis, both *gsb* and *D-six4* act upstream of *Poxm*, but the expression of *gsb* in the mesoderm neither depends on *D-six4* nor on *Poxm* (Figure 11).

The employment of Pax genes in myogenesis: a divergent or convergent process?

In *Drosophila*, as discussed here and in chapter 2, members of both *Pax1/9* and

Pax3/7 subfamilies are involved in myogenesis. In vertebrates, however, while *Pax3* and *Pax7* are crucial for myogenesis, *Pax1* and *Pax9* were only reported to affect the formation of cartilage and bone (Peters et al., 1999). Yet in both the somatic mesoderm of *Drosophila* and the somites of vertebrates, *Pax3/7* and *Pax1/9* are expressed in partially overlapping regions, with the expression domain of *Pax1/9* being more ventral (Borycki and Emerson, 1997; Peters et al., 1999; Fig. 1G-I). This implies that in spite of the changes in specific functions of *Pax1/9* and *Pax3/7*, the positional information that regulates their expressions may be conserved during evolution. If this is true, the involvement of Pax genes in myogenesis of both *Drosophila* and vertebrates could be a consequence of their upstream regulatory networks being conserved in a way that even though individual components of these networks may not be homologs, the overall outcome is the activation of particular Pax genes in specific body compartments. Cells of these body compartments can then acquire similar or different fates in different organisms. In *Drosophila*, both Poxm and mesodermal Gsb expressing cells enter myogenesis, whereas in vertebrates only cells expressing *Pax3/7* are important for myogenesis. This makes the convergent model possible in which *Drosophila* and vertebrates employ Pax genes in their myogenic programs independently during evolution.

Alternatively, the involvement of *Pax1/9* and *Pax3/7* in myogenesis reflects one of their ancient functions. In *Drosophila*, members from both subfamilies still retain this function, but later on, during the evolution of vertebrates, as organisms become more complex, gene functions were further specified so that genes previously involved in the same developmental processes may adopt related but distinct functions. Thus, although *Pax1/9* and *Pax3/7* both control the development of tissues derived from somites, only *Pax3/7* are important for myogenesis. In this case, vertebrate *Pax1/9* and *Pax3/7* gain their specific functions through a divergent process. Interestingly, phylogenetic analysis showed that *Pax1/9* and *Pax3/7* are more closely related and there is a basal dichotomy between *Pax1/9* plus *Pax3/7* and the other *Pax* genes (Breitling and Gerber, 2000). This implies that *Pax1/9* and *Pax3/7* are derived from a common ancestor through gene duplication. However, it is not clear when this duplication happened. Up to now, members of both subfamilies are found only in *Arthropoda*, *Deuterostomia* and *Nematoda* (according to Pfam database), from the phylogenetic tree, it is likely that this duplication happened before the separation of *Deuterostomia* and *Ecdysozoa* (Fig. 12; Noll, 1993). If this is the case, why are

Pax1/9 and *Pax3/7* not found in the other organisms having a common ancestor with *Arthropoda*? Since it is not likely that all of them have lost *Pax1/9* and *Pax3/7,* most probably it is because the available data is still incomplete. Thus, the proposed ancient function of *Pax1/9* and *Pax3/7* can only be deduced when more data is collected and their functions in many more organisms are known.

References

1. Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev*. 7, 1325-1340.

2. Bate, M., Rushton, E. and Currie, D. (1991). Cells with persistent *twist* expression are the embryonic precursors of adult muscles in *Drosophila*. *Development* 113, 79-89.

3. Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster*, Vol. 2 (ed. M. Bate and A. Martinez Arias), pp. 1013 - 1090. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

4. Baylies, M.K., and Bate, M. (1996). *twist*: a myogenic switch in *Drosophila*. *Science* 272**,** 1481-1484.

5. Baylies, M.K., Martinez Arias, A. and Bate, M. (1995). *wingless* is required for the formation of a subset of muscle founder cells. *Development* 121, 3829-3837.

6. Baylies, M.K., Bate, M. and Ruiz Gomez, M. (1998). Myogenesis: a view from *Drosophila*. *Cell* 93, 921-927.

7. Baylies, M.K. and Michelson, A.M. (2001). Invertebrate myogenesis: looking back to the future of muscle development. *Curr. Opin. Genet. De*v. 11, 431-439.

8. Beiman, M., Shilo, B-Z. and Volk, T. (1996). Heartless, a *Drosophila* FGF receptor homolog, is essential for cell migration and establishment of several mesodermal lineages. *Genes Dev*. 10, 2993-3002.

9. Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* 118, 719-729.

10. Bodmer, R., Jan, L.Y. and Jan, Y.N. (1990). A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation of *Drosophila*. *Development* 110, 661-669.

11. Borycki, A.G. and Emerson, C.P. (1997). Muscle determination: another key player in myogenesis? *Curr. Biol.* 7, R620-623.

12. Breitling, R. and Gerber, J.K. (2000). Origin of the paired domain. *Dev. Genes. Evol.* 210, 644-650.

13. Buckingham, M. and Relaix, F. (2007). The role of *Pax* genes in the development of tissues and organs: *Pax3* and *Pax7* regulate muscle progenitor cell functions. *Annu. Rev. Cell Dev. Biol.* 23, 645-673.

14. Chen, J. (2007). Analysis of the transcriptional regulation and developmental functions of *Poxm* in *Drosophila* melanogaster. Ph.D. thesis. University of Zürich.

15. Chen, R., Amoui, M., Zhang, Z. and Mardon, G. (1997). Dachshund and Eyes absent proteins form a complex and function synergistically to induce ectopic eye development in *Drosophila*. *Cell* 91, 893-903.

16. Clark, I.B.N., Boyd, J., Hamilton, G., Finnegan, D.J. and Jarman, A.P. (2006). *D*-six4 plays a key role in patterning cell identities deriving from the *Drosophila* mesoderm. *Dev. Biol.* 294, 220-231.

17. Crossley, A.C. (1978). The morphology and development of the *Drosophila* muscular system. In *The Genetics and Biology of Drosophila*. Vol. 2b (ed. M. Ashburner and T. R. F. Wright), pp. 499-560. New York: Academic Press.

18. Czerny, T., Halder, G., Kloter, U., Souabni, A., Gehring, W.J. and Busslinger, M. (1999). *twin of eyeless*, a second *Pax-6* gene of *Drosophila*, acts upstream of *eyeless* in the control of eye development. *Mol. Cell* 3, 297-307.

19. Duan, H., Zhang, C., Chen, J., Sink, H., Frei, E. and Noll, M. (2007). A key role of *Pox meso* in somatic myogenesis of *Drosophila*. *Development* 134, 3985-3997.

20. Duman-Scheel, M., Li, X., Orlov, I., Noll, M. and Patel, N.H. (1997). Genetic separation of the neural and cuticular patterning functions of *gooseberry*. *Development* 124, 2855-2865.

21. Dunin Borkowski, O.M., Brown, N.H. and Bate, M. (1995). Anterior-posterior subdivision and the diversification of the mesoderm in *Drosophila*. *Development* 121, 4183-4193.

22. Frasch, M. (1995). Induction of visceral and cardiac mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature* 374, 464-467.

23. Gallardo, M.E., Lopez-Rios, J., Fernaud-Espinosa, I., Granadino, B., Sanz, R., Ramos, C., Ayuso, C., Seller, M.J., Brunner, H.G., Bovolenta, P. and Rodríguez de Córdoba, S. (1999). Genomic cloning and characterization of the human homeobox gene SIX6 reveals a cluster of SIX genes in chromosome 14 and associates SIX6 hemizygosity with bilateral anophthalmia and pituitary anomalies. *Genomics* 61, 82-91.

24. Gisselbrecht, S., Skeath, J.B., Doe, C.Q. and Michelson, A.M. (1996). *heartless* encodes a fibroblast growth factor receptor (DFR1/DFGF-R2) involved in the directional migration of early mesodermal cells in the *Drosophila* embryo. *Genes Dev*. 10, 3003-3017.

25. Gutjahr, T., Patel, N., Li, X., Goodman, C., and Noll, M. (1993). Analysis of the gooseberry locus in Drosophila embryos: gooseberry determines the cuticular pattern and activates gooseberry neuro. *Development* 118, 21-31.

26. Grifone, R., Demignon, J., Houbron, C., Souil, E., Niro, C., Seller, M.J., Hamard, G., and Maire, P. (2005). Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. *Development* 132, 2235-2249.

27. Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W.J. (1998). *Eyeless* initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development. *Development* 125, 2181-2191.

28. He, H. (2007). Coupling of redundant *gooseberry neuro* and *gooseberry* functions, an evolutionary strategy against an intrinsic haploinsufficiency of *gooseberry*? Ph.D. thesis. University of Zürich.

29. Hill, R.E., Favor, J., Hogan, B.L., Ton, C.C., Saunders, G.F., Hanson, I.M., Prosser, J., Jordan, T., Hastie, N.D. and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* 354, 522-525.

30. Jiao, R., Daube, M., Duan, H., Zou, Y., Frei, E. and Noll, M. (2001). Headless flies generated by developmental pathway interference. *Development* 128, 3307-3319.

31. Kardon, G., Heanue, T.A. and Tabin, C.J. (2004). *Modularity in Development and Evolution* (eds Schlosser, G. and Wagner, G.P.). 59-80 (Univ. Chicago Press, Chicago and London).

32. Kiehart, D.P. and Feghali, R. (1986). Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol*. 103, 1517-1525.

33. Kirby, R.J., Hamilton, G.M., Finnegan, D.J., Johnson, K.J. and Jarman, A.P. (2001). *Drosophila* homolog of the myotonic dystrophy-associated gene, SIX5, is required for muscle and gonad development. *Curr. Biol.* 11, 1044-1049.

34. Lee H.-H. and Frasch, M. (2000). Wingless effects mesoderm patterning and ectoderm segmentation events via induction of its downstream target *sloppy paired*. *Development* 127, 5497-5508.

35. Li, X. and Noll, M. (1993). Role of the *gooseberry* gene in *Drosophila* embryos: maintenance of *wingless* expression by a *wingless-gooseberry* autoregulatory loop. *EMBO J.* 12, 4499-4509.

36. Meadows, L.A., Gell, D., Broadie, K., Gould, A.P. and White, R.A. 1994. The cell adhesion molecule, connectin, and the development of the *Drosophila* neuromuscular system. *J. Cell Sci.* 107, 321-328.

37. Noll, M. (1993). Evolution and role of *Pax* genes. *Curr. Opin. Genet. Dev*. 3, 595-605.

38. Oliver, G., Mailhos, A., Wehr, R., Copeland, N.G., Jenkins, N.A. and Gruss, P. (1995). *Six3*, a murine homologue of the *sine oculis* gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* 121, 4045-4055.

39. Peters, H., Wilm, B., Sakai, N., Imai, K., Maas, R. and Balling, R. (1999). Pax1 and Pax9 synergistically regulate vertebral column development. *Development* 126, 5399-5408.

40. Pignoni, F., Hu, B., Zavitz, K.H., Xiao, J., Garrity, P.A. and Zipursky, S.L. (1997). The eye specification proteins So and Eya form a complex and regulate multiple steps in *Drosophila* eye development. Cell *91*, 881-891.

41. Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R. and Leptin, M. (1997). Control of cell fates and segmentation in the *Drosophila* mesoderm. *Development*

124, 2915-2922.

42. Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* 59, 1189-1202.

43. Ruiz-Gómez, M., Romani, S., Hartmann, C., Jäckle, H. and Bate, M. (1997). Specific muscle identities are regulated by *Krüppel* during *Drosophila* embryogenesis. *Development* 124, 3407-3414.

44. Shen, W. and Mardon, G. (1997). Ectopic eye development in *Drosophila* induced by directed *dachshund* expression. *Development* 124, 45-52.

45. Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E. and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila.* *Nature* 372, 783-786.

46. Thisse, B., Stoetzel, C., Gorostiza, T.C. and Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J*. 7, 2175-2183.

47. Wallis, D.E., Roessler, E., Hehr, U., Nanni, L., Wiltshire, T., Richieri-Costa, A., Gillessen-Kaesbach, G., Zackai, E.H., Rommens, J. and Muenke, M. (1999). Mutations in the homeodomain of the human SIX3 gene cause holoprosencephaly. *Nat Genet.* 22, 196-198.

48. Yin, Z. and Frasch, M. (1998). Regulation and function of *tinman* during dorsal mesoderm induction and heart specification in *Drosophila*. *Dev. Genet*. 22, 187-200.

49. Zhang, Y., Ungar, A., Fresquez, C. and Holgrem, R. (1994). Ectopic expression of either *Drosophila gooseberry-distal* or *proximal* gene causes alterations of cell fate in the epidermis and central nervous system. *Development* 120, 1151-1161.

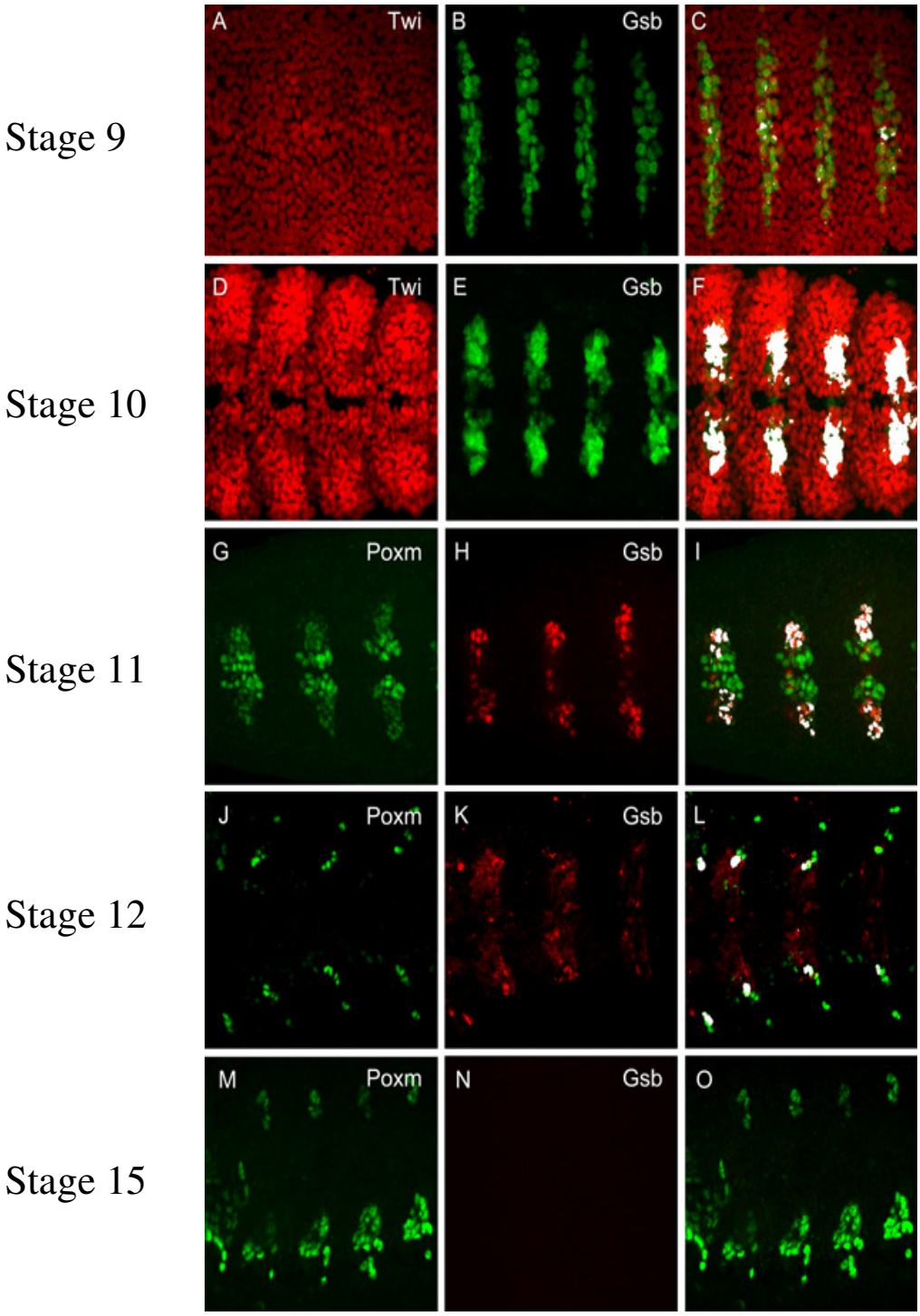


Fig. 1. Gsb is expressed in the mesoderm from stage 10 to stage 12. Wild-type embryos at stages 9 (A-C), 10 (D-F), 11 (G-I), 12 (J-L), and 15 (M-O) stained for Twi (A, D), Gsb (B, E, H, K, N), and Poxm (G, J, M) are shown. Cells in which Gsb and Twi (C, F), or Gsb and Poxm (I, L, O) colocalize are highlighted in white by the use of the image processing program ImageJ. Either ventral (A-L) or lateral views (M-O) of embryos are shown with their anterior to the left.

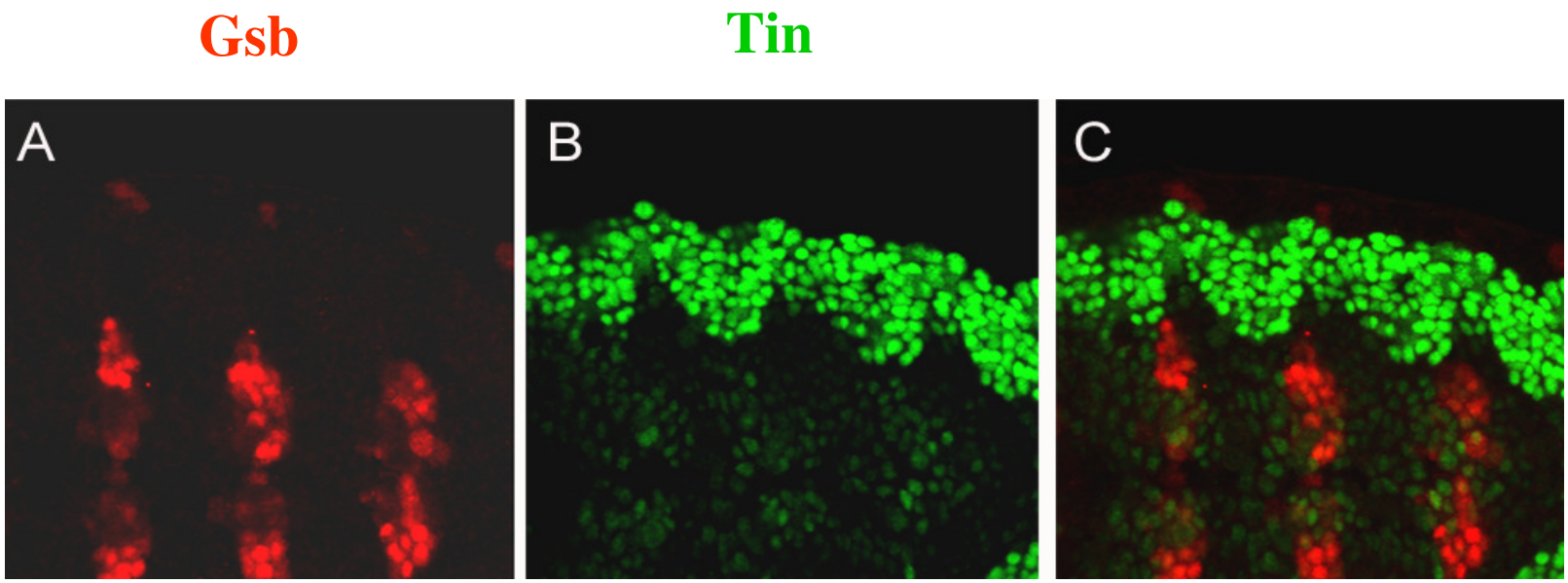


Fig. 2. Gsb is not expressed in the dorsal mesoderm where Tin is expressed strongly. Wild-type embryos at stage 11 stained for Gsb (A) and Tin (B) are shown. Colocalization analysis is performed by the use of the image processing program ImageJ the result of which is shown in panel C. Ventral views of the embryos with their anterior to the left are shown.

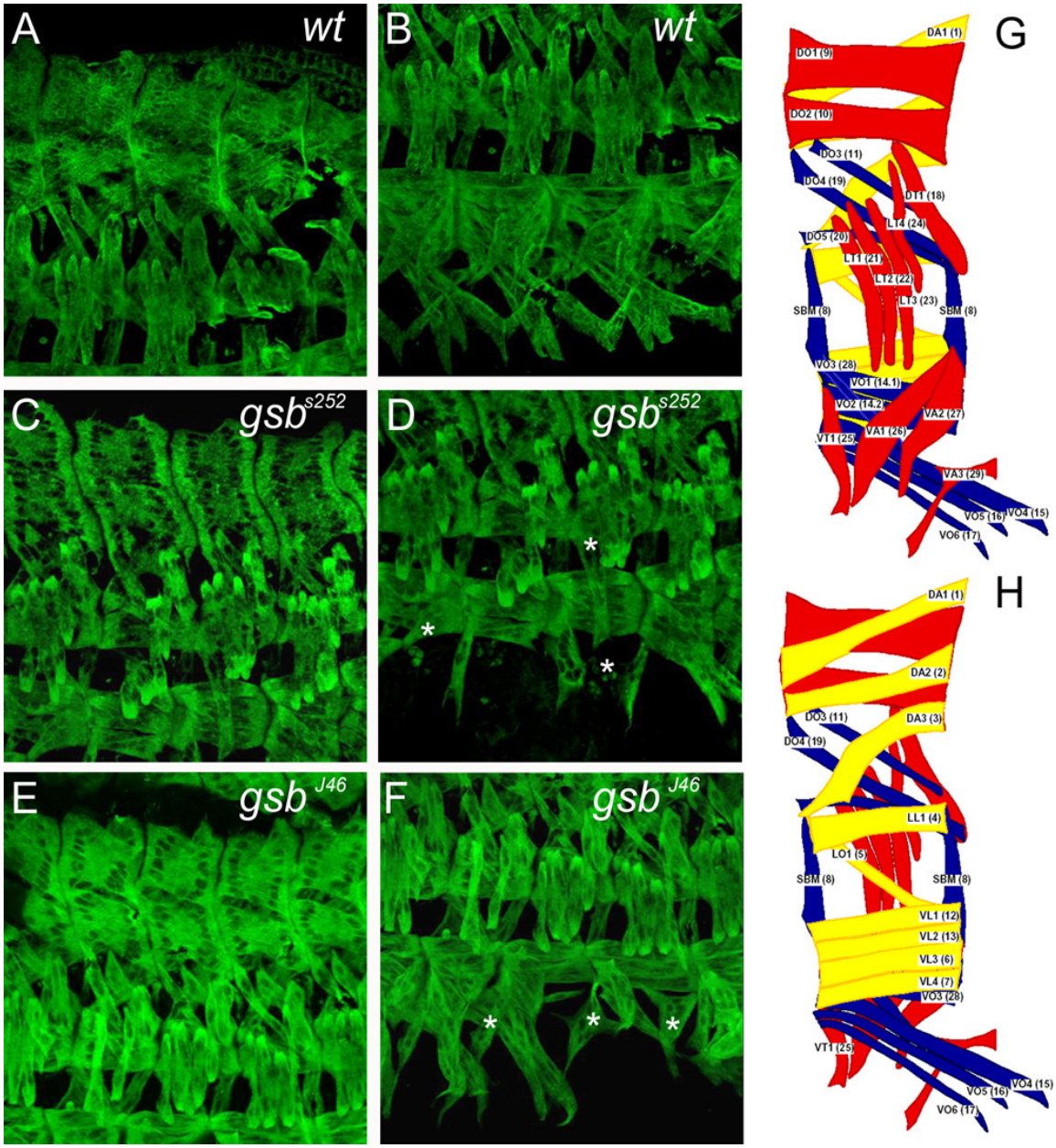


Fig. 3. The pattern of somatic body wall muscles is severely affected in *gsb* mutants. Dorsal to lateral (A, C, E) and lateral to ventral (B, D, F) muscles of *wt* (A, B), gsb^{s252} (C, D), and gsb^{J46} (E, F) stage 16 embryos were visualized by the use of an anti-MHC antiserum. Schematic external view (G) and internal view (H) of larval muscles in abdominal segments A2-A7 (Ruiz-Gómez et al., 1997), with external muscles in red and more internal muscles in blue and yellow; muscles are designated and numbered according to Bate (Bate, 1993) and in parentheses according to Crossley (Crossley, 1978). Segments are oriented with anterior to the left and dorsal up. Asterisks mark places where muscles are abnormal or missing.

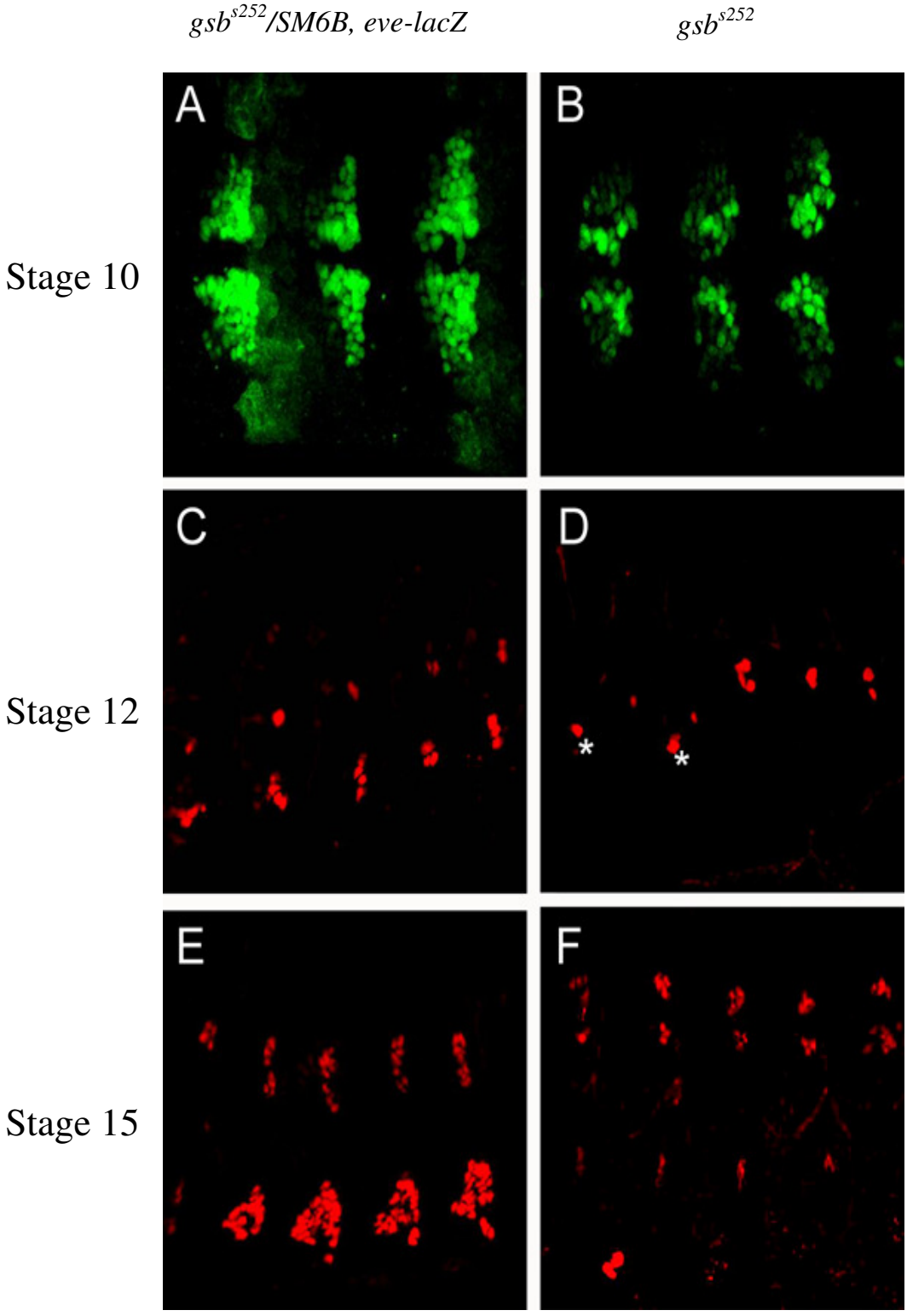


Fig. 4. Poxm expression is affected in *gsb* mutants. *gsb*^{s252}*/SM6B, eve-lacZ* (A, C, E) and *gsb*^{s252} (B, D, F) embryos at stages 10 (A, B), 12 (C, D), and 15 (E, F) stained for Poxm are shown. Ventral (A, B) or lateral views (C-F) of embryos with their anterior to the left are shown. Asterisks in D indicate the expression of Poxm in the ventral region of thoracic segments.

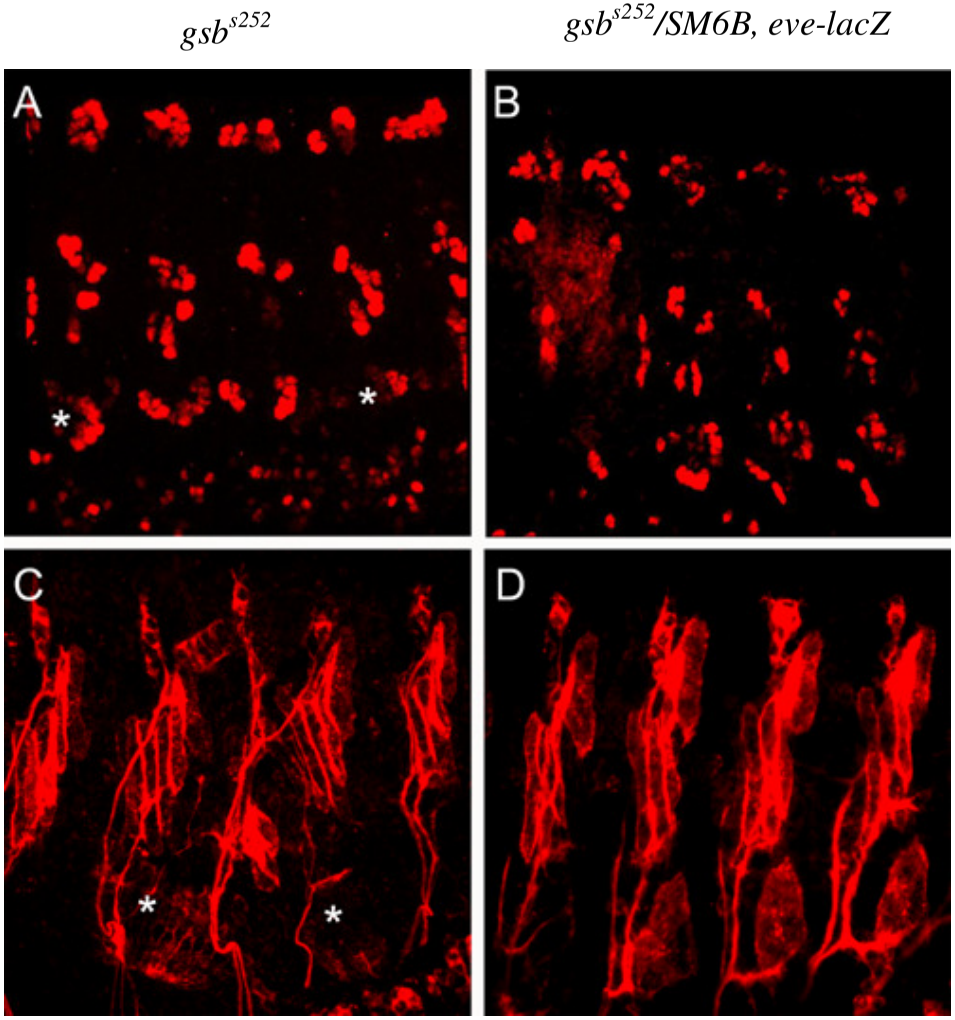


Fig. 5. The expression of Kr and Connectin is affected in *gsb* mutants. *gsb*^{s252} (A, C) and *gsb*^{s252}*/SM6B, eve-lacZ* (B, D) embryos at stage 15 stained for Kr (A, B) and Connectin (C, D) are shown. The embryos are oriented with their anterior to the left and dorsal side up. Asterisks in A and C indicate where Kr or Connectin expression is abnormal.

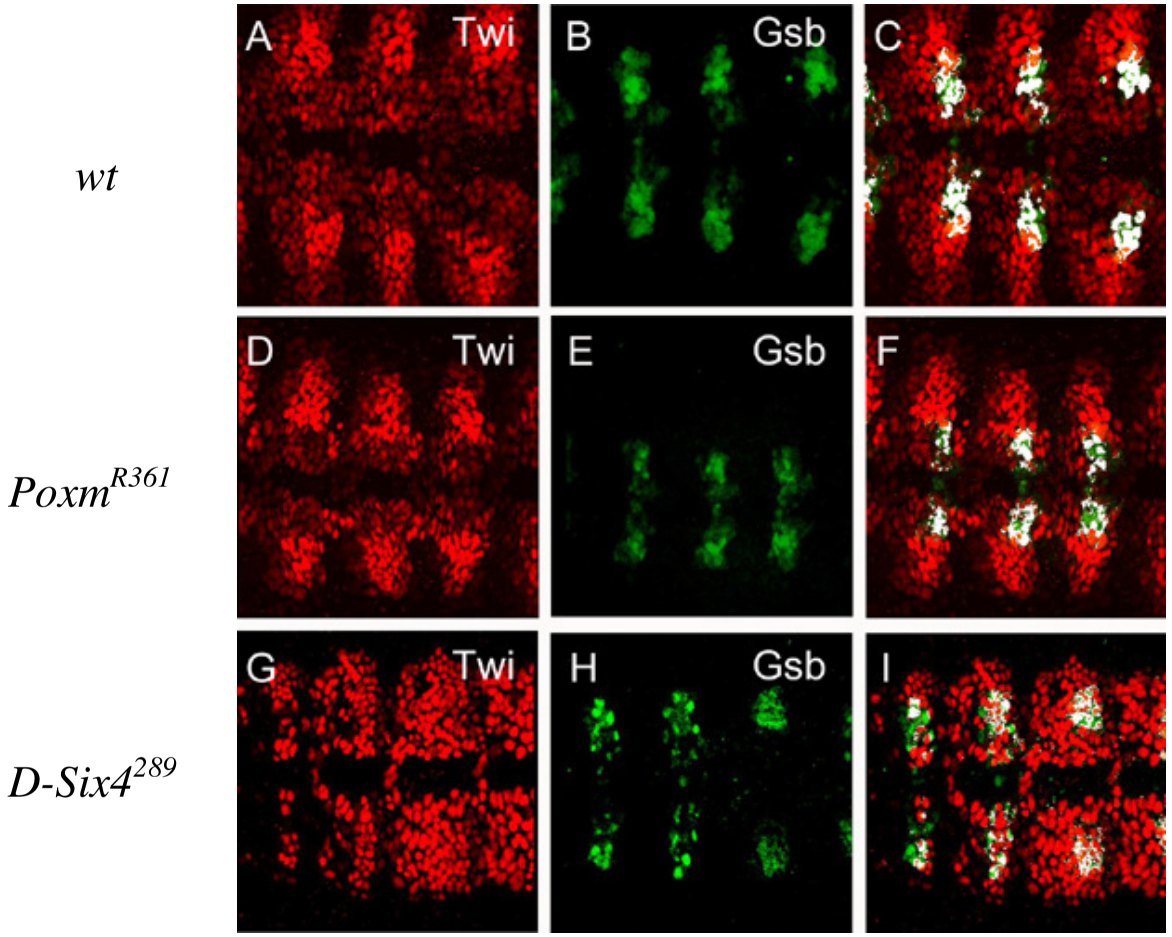
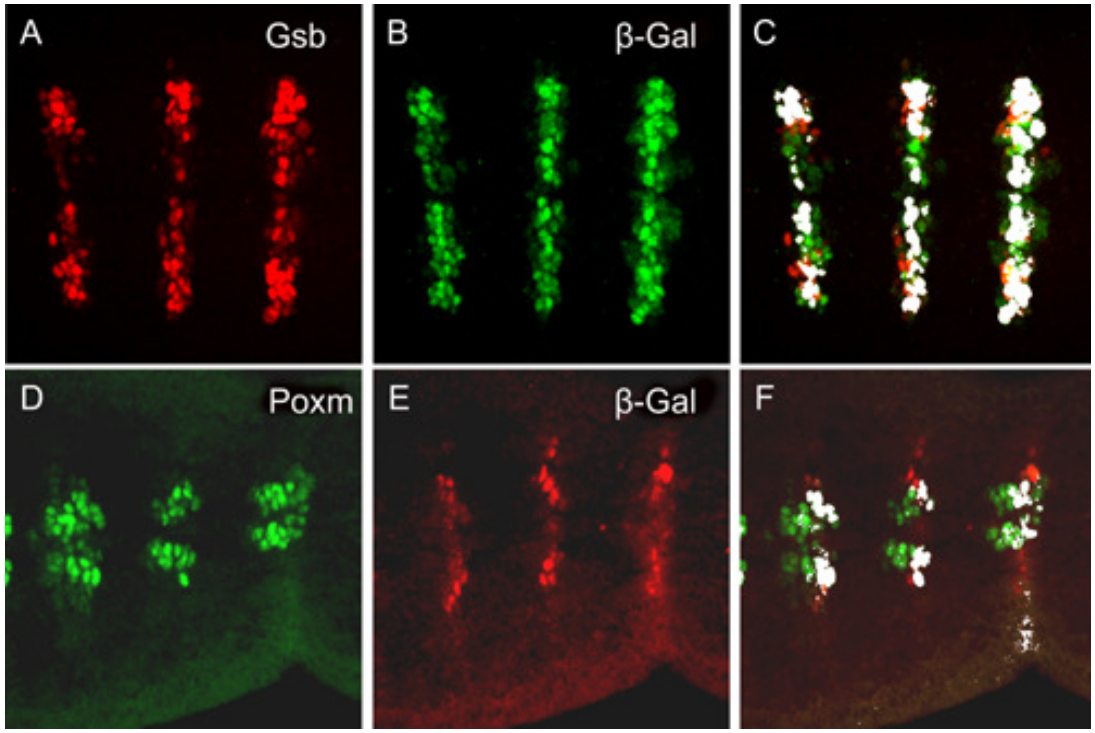


Fig. 6. Gsb expression in the mesoderm is not affected in *Poxm* and *D-Six4* mutants. Wild-type (A-C), *Poxm*^{R361} (D-F), and *D-Six4*^{289} (G-I) embryos at stage 11 stained for Twi (A, D, G) and Gsb (B, E, H) are shown. Colocalization analysis is performed by the use of the image processing program ImageJ and the results are shown in panels C, F, and I. The embryos are oriented with their anterior to the left and dorsal side up.



gsb^{J46}*/CyO, Kr-GFP*

gsb^{J46}*/ gsb*^{s252}

Fig. 7. The regulation of *Poxm* by *gsb* is mainly a non-cell-autonomous process. *gsb*^{J46}*/CyO, Kr-GFP* (A-C), and *gsb*^{J46}*/ gsb*^{s252} (D-F) embryos at stage 11 stained for Gsb (A), β -Galactosidase (B, E), and Poxm (D) are shown. Colocalization analysis is performed by the use of the image processing program ImageJ the results of which are shown in panels C and F. Ventral views of embryos with their anterior to the left are shown.

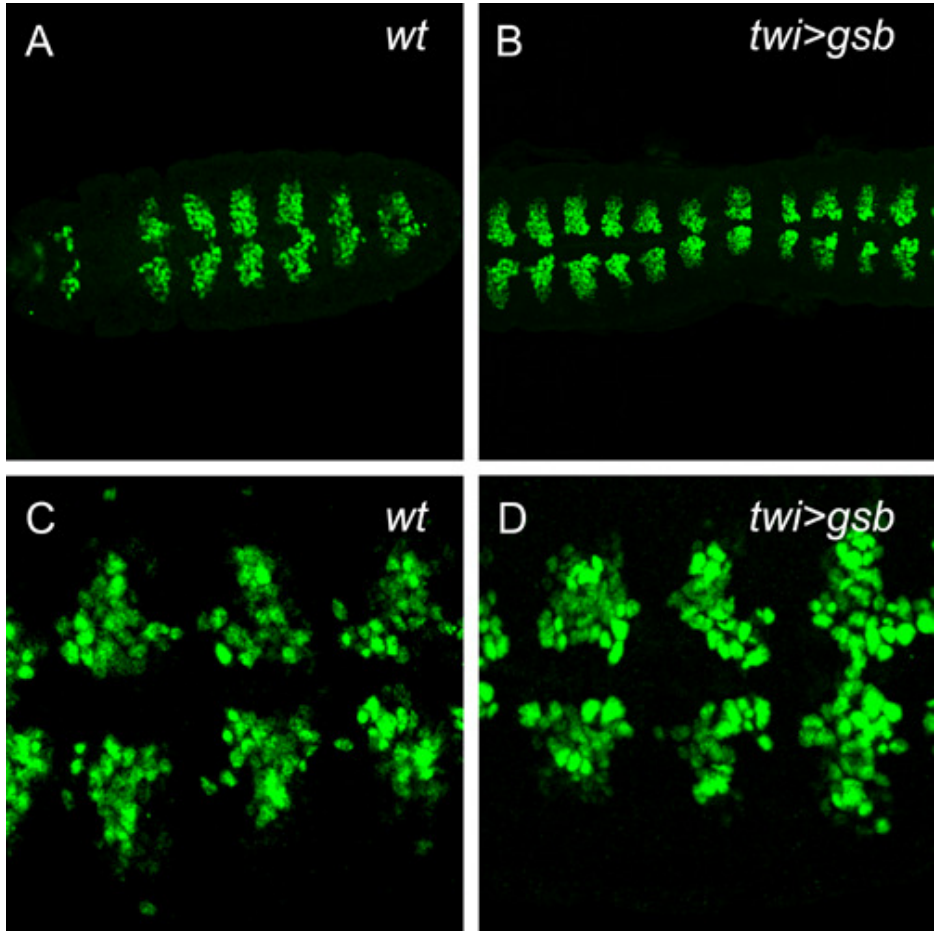


Fig. 8. Early Poxm expression is not affected by ubiquitous overexpression of *gsb* in the mesoderm. Wild-type (A, C) and *twi-Gal4/UAS-gsb* (B, D) embryos at early (A, B) and late (C, D) stage 11 stained for Poxm are shown. Ventral views of the embryos with their anterior to the left are shown.

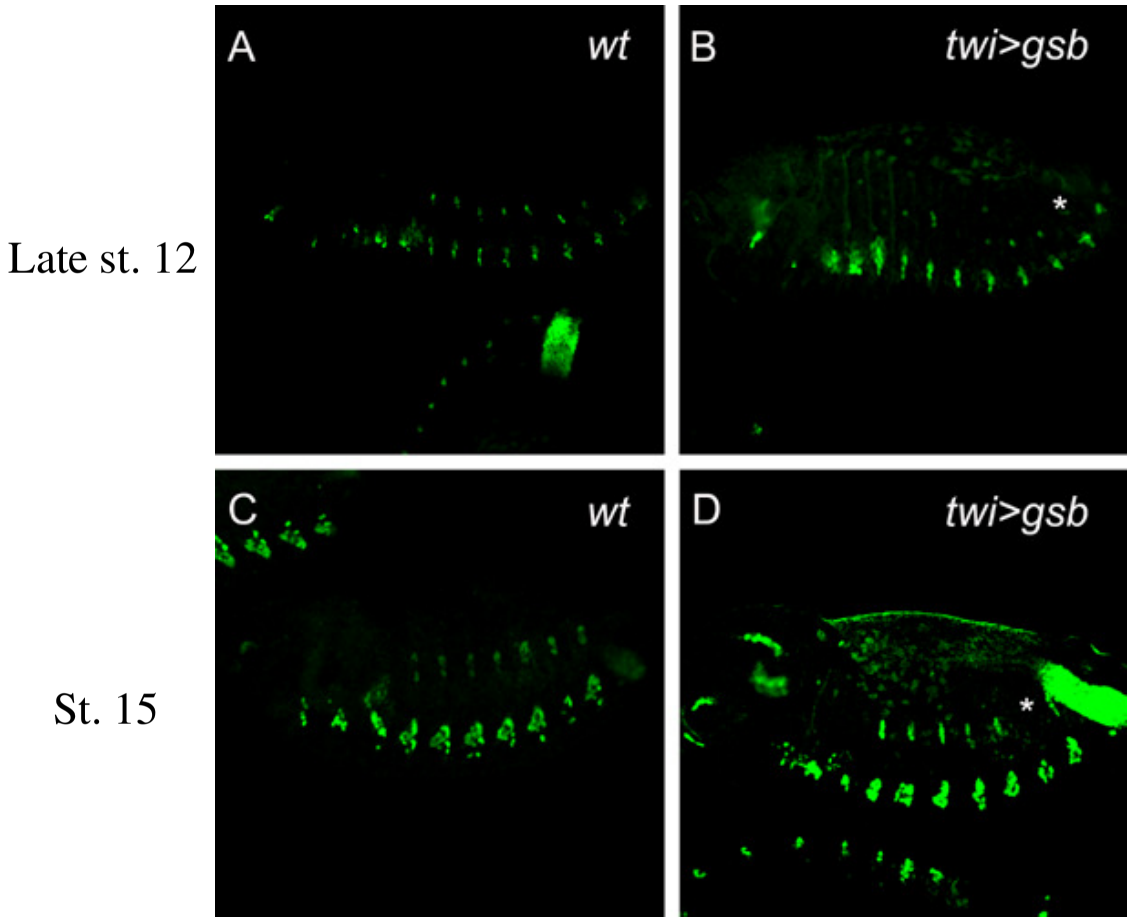


Fig. 9. Late Poxm expression is affected when *gsb* is ectopically overexpressed. Wild-type (A, C) and *twi-Gal4/UAS-gsb* (B, D) embryos at late stage 12 (A, B) and stage 15 (C, D) are stained for Poxm. The embryos are oriented with their anterior to the left and dorsal side up. Asterisks mark places where Poxm expression is abolished.

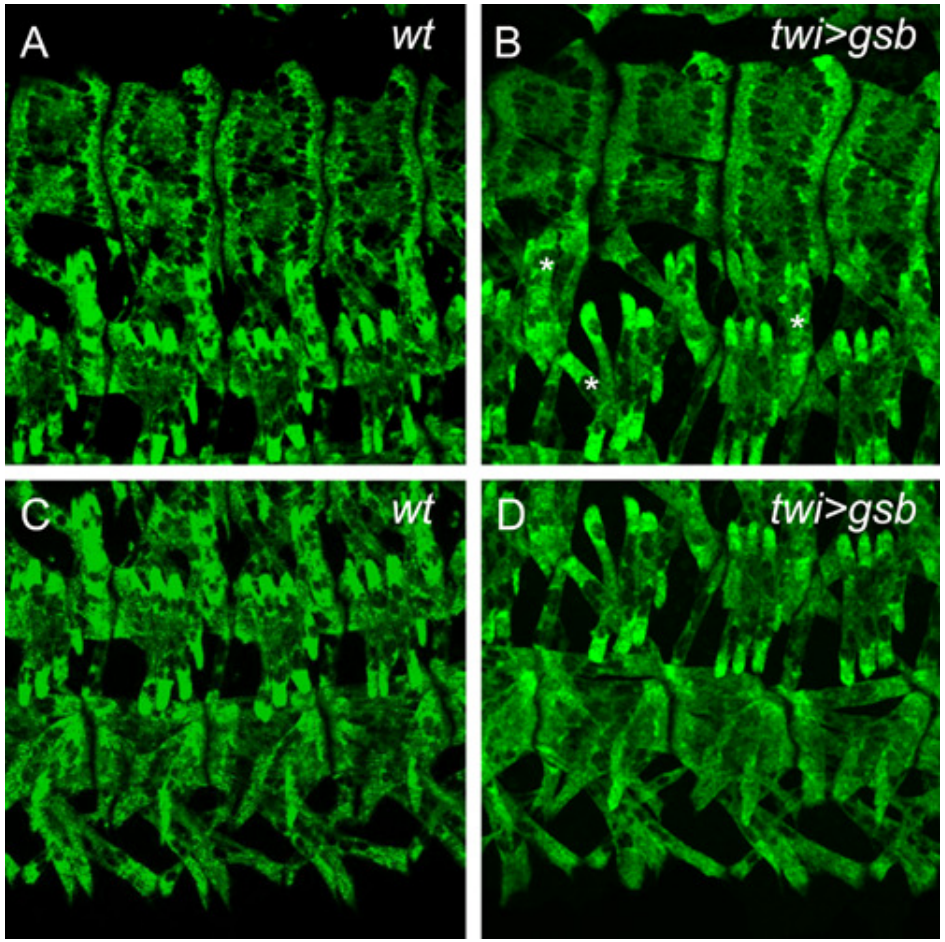


Fig. 10. The pattern of somatic body wall muscles is affected when *gsb* is ectopically overexpressed. Muscle patterns of wild-type (A, C) and *twi-Gal4/UAS-gsb* (B, D) stage 16 embryos were visualized by staining with an anti-MHC antiserum. The embryos are oriented with their anterior to the left and dorsal side up. Asterisks mark places where abnormal muscles are observed.

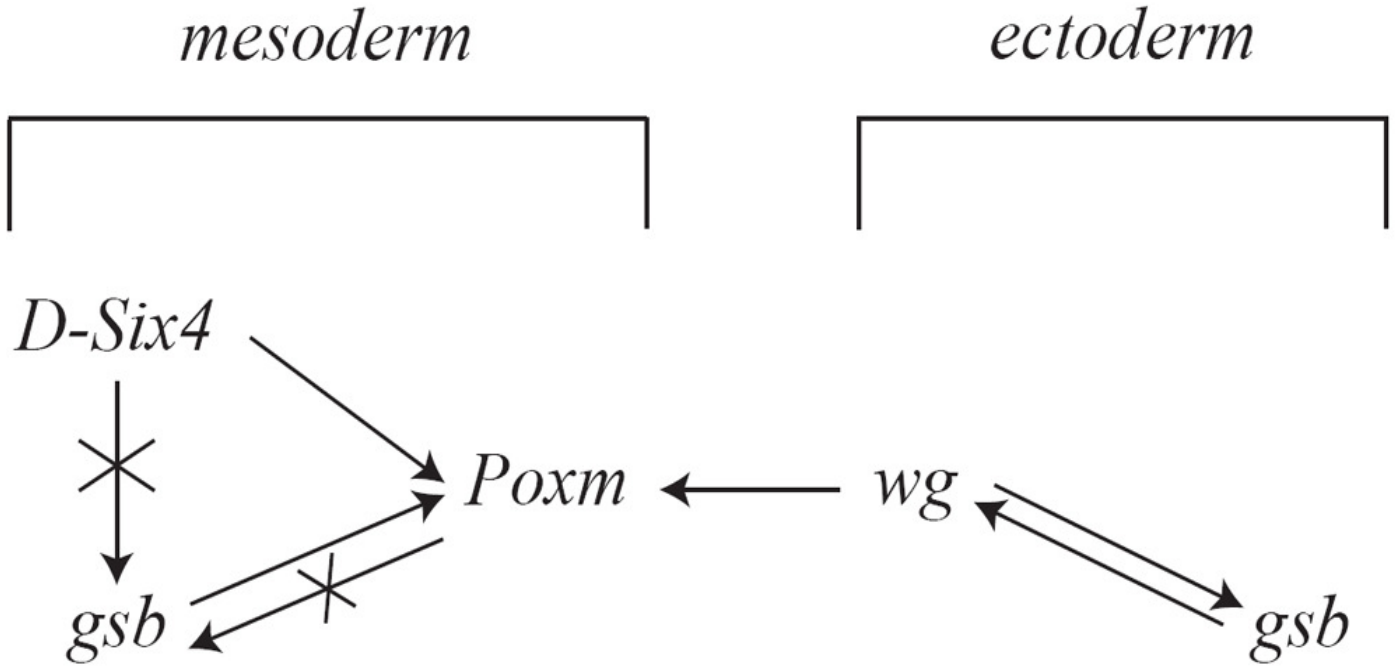


Fig. 11. Model of the "*Pax-Six* gene network" in *Drosophila* myogensis. Gene activation is indicated by arrows. Arrows with small crosses indicate that these activations do not exist. For detailed explanation, see text.

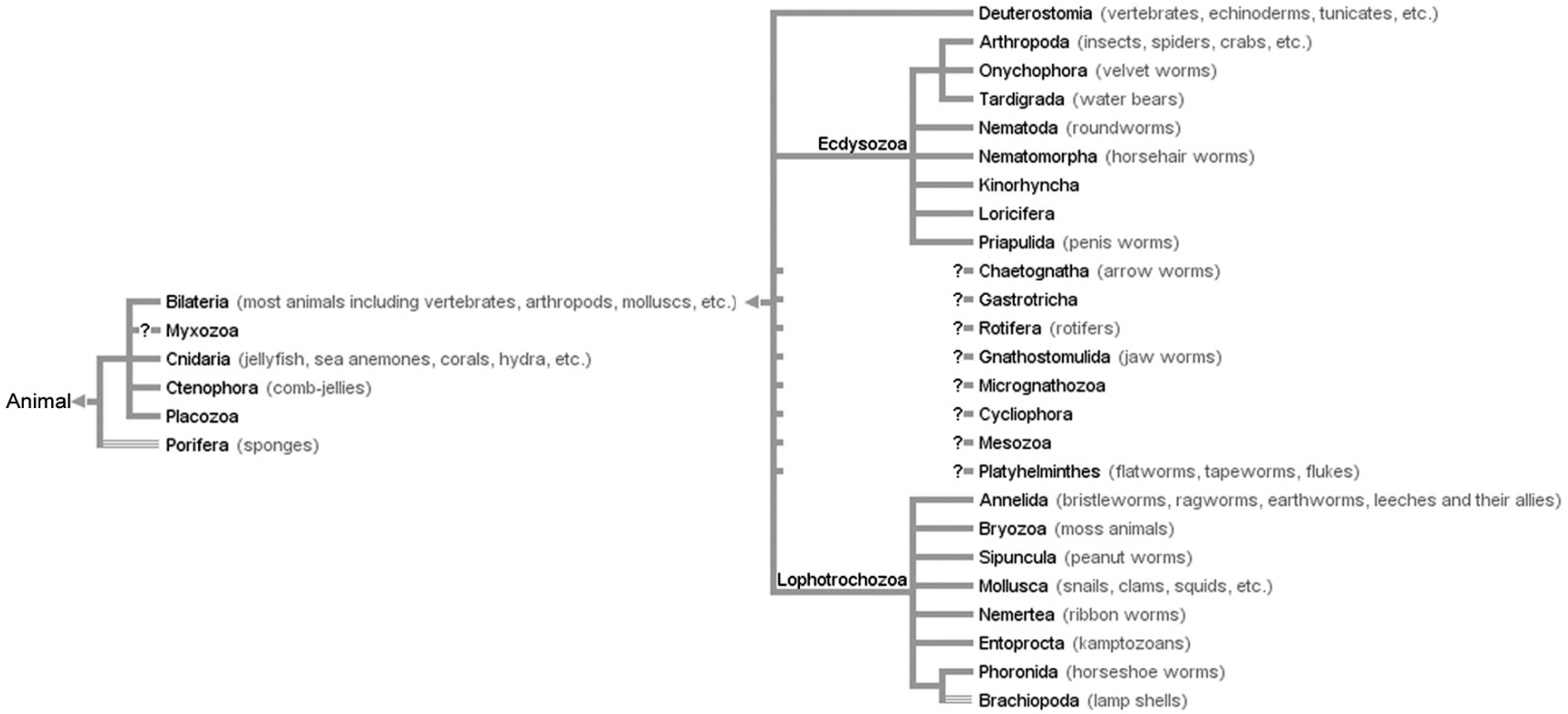


Fig. 12. Phylogenetic tree of organisms. Adapted from *The Tree of Life Web Project*, http://tolweb.org.

Chapter 4

The Repression of *Poxm* by *Dpp* Signaling

Summary

Understanding the temporal and spacial control of gene expression will help us to decipher the gene networks regulating developmental processes. The *Pax 1/9* homolog *Pox meso* (*Poxm*) plays a key role in *Drosophila* embryonic myogenesis. It was shown to be repressed by Dpp signaling nearly 14 years ago. However, the mechanism by which this repression is achieved still remains a mystery. Here, I show that it is mediated by the Dpp receptor TKV and the R-SMAD MAD, but not the co-repressor Shn. I have identified a 280 bp fragment, *um2I&2II*, in the upstream regulatory region of *Poxm* that not only responds to Dpp signaling in wild-type embryos to restrict *Poxm* expression in the dorsolateral mesoderm, but also confers repression by Dpp when ectopically expressed in the ventral ectoderm. These results raise the possibility that molecular mechanisms independent of Shn may be employed in Dpp dependent repression.

Introduction

The transforming growth factor β (TGFβ) signaling pathway is important for many biological processes in virtually all tissues in organisms, from fly to human (reviewed by Massagué, J., 1998). It functions by activating as well as repressing target genes. In vertebrates, several molecular mechanisms have been described to mediate the repression by TGFβ signaling (Chen et al., 2002; Kang et al., 2003). In these cases, the Smad proteins form complexes with other transcriptional co-regulators and bind to specific *cis*-regulatory elements of the repressed target genes. In *Drosophila*, signaling by the TGFβ superfamily member Decapentaplegic (Dpp) is also important

for many developmental processes (St. Johnston et al., 1990). Dpp activates many of its targets indirectly by repressing *brinker* (*brk*), which functions as a default repressor to repress Dpp targets in the absence of the ligand (reviewed by Affolter et al., 2001). The cells perceive the presence of Dpp upon its interaction with the receptors Punt and Thickveins (TKV). Then, the activated receptors phosphorylates Mothers-against-dpp (MAD) and the phosphorylated MAD (pMAD) subsequently forms a complex with Medea. This complex is translocated into the nucleus and the co-repressor Schnurri (SHN) is recruited after binding of the pMAD–Medea complex to the upstream regulatory regions of *brk* to repress its transcription (reviewed by Affolter and Basler, 2007). A silencer was characterized in the upstream regulatory region of *brk* to mediate transcriptional repression by Dpp (Müller et al., 2003), and the minimal DNA sequence element (SE) required to keep this silencer functional has been identified (Pyrowolakis et al., 2004). It was proposed that this SE serves as a *cis*-regulatory signature for Dpp-dependent repression without the involvement of cell type-specific transcription coregulators (Pyrowolakis et al., 2004).

Although *Poxm* was known to be repressed by Dpp signaling (Staehling-Hampton et al., 1994), the molecular mechanisms of this repression remained a mistery. In this chapter, I demonstrate that Dpp signaling represses *Poxm* expression through mesodermal intrinsic factors. Multiple components of the canonical Dpp signaling pathway are involved in this repression. However, unlike all previously reported targets repressed by Dpp signaling, *Poxm* is repressed by Dpp independent of the co-repressor Shn. In addition, I isolated a short Dpp dependent *cis*-regulatory element, *um2I&2II*, that is both necessary and sufficient for early *Poxm* expression. Comparing these results with previously characterized Dpp-dependent silencers may help us to further understand how Dpp signaling represses gene expression in different developmental processes.

Materials and Methods

P-element mediated transformation

P-element-mediated germ line transformation of *y w* flies was performed as previously described (Rubin and Spradling, 1982). For each construct, at least five independent lines were analyzed. Different lines from the same construct showed essentially the same expression patterns.

Immunohistochemistry and microscopy

The following primary antisera were used: rabbit anti-Poxm (Duan et al., 2007), rat anti-Gsb (Zhang et al., 1994), rabbit anti-MHC (myosin heavy chain; Kiehart and Feghali, 1986), rabbit anti-Twist (Roth et al., 1989), and rabbit anti-Galactosidase (Cappel). Embryos were fixed and stained as described previously (Gutjahr et al., 1993).

Muscle patterns were visualized after staining with anti-MHC. The fluorescent signals were amplified by tyramide signal amplification (TSA; kits #12 and #25 from Invitrogen), and embryos were analyzed with a Leica SP1 confocal microscope.

Fly stocks.

The following fly stocks were used:

y w,

twi-Gal4 (Bloomington stock 914),

dpp^{hr27} (Spencer et al., 1982),

dpp^{hr56} (Irish and Gelbart, 1987),

y w; prd-Gal4 (Xiao et al., 1996),

y w; UAS-dpp (Tracey et al., 2000),

y w; $UAS\text{-}tkv^{QD}$ (Nellen et al., 1996),

y w; UAS-dad (Raftery and Sutherland, 1999),

y w; UAS-mad (Raftery and Sutherland, 1999),

mad^{12} (Sekelsky et al., 1995),

Shn^{TD5}/CyO (Marty et al., 2000),

y w; um2-lacZ (Duan et al., 2007),

y w; um2I_2II-lacZ,

y w; um2I&2II-lacZ,

y w; um2δIδII-lacZ,

Drosophila erecta,

Drosophila pseudoobscura,

Drosophila virilis.

Results

The expression of Poxm is repressed by Dpp signaling

In dpp^{H61} embryos, the Poxm expression Poxm expands to the dorsal region where normally no Poxm protein can be detected (Staehling-Hampton et al., 1994). Since dpp^{H61} is a very strong allele, homozygous embryos of which fail to complete germ band extension but die with a strong ventralized phenotype and have no dorsal derived epidermis (St. Johnston et al., 1990), it is likely that the effect on Poxm expression is indirect and caused by the overall ventralization of these embryos. Therefore, two hypomorphic alleles, dpp^{hr27} and dpp^{hr56}, were used to test this possibility. dpp^{hr27} embryos show a moderate ventralized phenotype (Arora and Nüsslein-Volhard, 1992), whereas most of the abdominal segments in dpp^{hr56} embryos are not ventralized (Wharton et al., 1993). In wild-type stage 11 embryos, Poxm is expressed in the ventral and lateral regions of the mesoderm with a gradient along the ventral-dorsal and the posterior-anterior axes (Fig. 1A; Duan et al., 2007). In both dpp^{hr27} and dpp^{hr56} embryos, the expression of Poxm extends to the dorsal regions (indicated by arrows in Fig. 1B, D). Therefore, ventralization of the embryos is not a prerequisite for the dorsal extension of Poxm expression in *dpp* mutants. In addition, consistent with a previous report (Staehling-Hampton et al., 1994), ectopic expression of *dpp* in every other segment of the ectoderm through *prd-Gal4>UAS-dpp* or ubiquitously in the mesoderm by *twi-Gal4>UAS-dpp* suppresses Poxm expression (Figs. 1C and 3C).

Dpp signaling is activated within the mesoderm to repress Poxm expression

When Dpp is ectopically expressed in the ectoderm, it may affect genes in the ectoderm, which in turn influence the mesoderm and repress *Poxm*. Alternatively, as a secreted morphogen Dpp may directly affect mesodermal cells (Affolter et al., 2001). Thus, repression of *Poxm* in the mesoderm may result from the activation of Dpp signaling in mesodermal cells that bind the Dpp ligands secreted from ectodermal cells. To test these possibilities, a constitutively activated form of the Dpp receptor, TKV^{Q253D} (Nellen et al., 1996), was overexpressed either in the ectoderm under the control of *prd-Gal4*, or in the mesoderm under the control of *twi-Gal4*. Because TKV is a membrane-bound receptor which acts cell-autonomously (Nellen et al., 1994; Penton et al., 1994), Dpp signaling is activated only in cells

expressing TKV^{Q253D}. Interestingly, while *Poxm* was repressed by ectopic Dpp signaling in the mesoderm (Fig. 2B), when ectopically activated only in the ectoderm, it cannot repress *Poxm* (Fig. 2A). These results indicate that Dpp signaling acts on *Poxm* within the mesoderm.

Components of the canonical Dpp signaling pathway are involved in the repression of *Poxm*

To understand how repression of *Poxm* by Dpp is achieved, the functions of canonical Dpp signaling pathway components were analyzed. As shown above, overexpression of the constitutively activated typeI-Dpp receptor, TKV, in the mesoderm is able to repress *Poxm* expression. Because the activated TKV performs its functions by phosphorylating the nuclear component MAD, expression of Poxm in *mad* mutants was examined. Unlike in wild-type embryos (Fig. 1C), ectopically expressed Dpp under the control of *prd-Gal4* cannot repress *Poxm* completely in zygotic mad^{12} mutant embryos (Fig. 3F). This suggests that MAD is also involved in the repression of *Poxm* by Dpp. However, comparing Poxm expression between neighboring segments, as *prd-Gal4* is expressed only in every other segment, reveals that *Poxm* can still be repressed in the absence of zygotic MAD, probably due to the remaining activity of maternal MAD. On the other hand, overexpression of *mad* in the mesoderm by *twi-Gal4* cannot repress *Poxm* (Fig. 3E) and therefore does not mimic the effect of ectopically activated Dpp signaling. This result shows that the amount of activated MAD instead of MAD protein itself is the limiting factor for Dpp signaling to be activated in the ventral and lateral regions of the mesoderm.

To confirm that MAD is involved in the repression of *Poxm* by Dpp, the role of the inhibitory Smad, DAD, was tested. DAD antagonizes Dpp signaling by inhibiting TKV-induced MAD phosphorylation and hence blocking hetero-oligomerization and nuclear translocation of MAD (Inoue et al., 1998). When DAD is overexpressed in the mesoderm under the control of *twi-Gal4*, Poxm expression extends to the dorsal mesoderm (Fig. 3B) like in *dpp* mutants (Fig. 1B, D; Staehling-Hampton et al., 1994). This phenotype was rescued when, in addition to DAD, Dpp was overexpressed (Fig. 3D). Conversely, the repression of *Poxm* by ectopically expressed Dpp (Fig. 3C) is rescued by overexpressing DAD at the same time (Fig. 3D). These results show that DAD regulates the expression of Poxm by antoganizing Dpp signaling.

Early Poxm expression is not affected in *shn* mutants

Shn has been considered a key factor for Dpp-dependent repression (Pyrowolakis et al., 2004). Therefore, I examined the functions of Shn in the repression of Poxm by Dpp. In these experiments, the null allele shn^{TD5} was used (Grieder et al., 1995). Consistent with previous results that Shn is required for the repression of another Pax gene, *gsb*, in the dorsal ectoderm (Pyrowolakis et al., 2004), the expression of Gsb expands dorsally in homozygous shn^{TD5} embryos (Fig. 4C, D). However, Poxm expression was not affected in the absence of *shn* (Fig. 4A, B). These results imply that the repression of Poxm by Dpp signaling in the dorsal mesoderm is *shn* independent.

Isolation of the Dpp-dependent silencer in the upstream regulatory region of *Poxm*

As discussed above, Dpp and multiple components of the canonical Dpp singling pathway are required for the repression of *Poxm*. To determine whether this repression is direct, I searched for a Dpp-dependent silencer in the upstream regulatory region of *Poxm*. Previous analysis showed that a 1.8 kb enhancer, *um2*, is important for the early expression of *Poxm* (Duan et al., 2007; Chen, 2003). To locate the Dpp-dependent silencer in this early enhancer, I dissected it further (Fig. 5). Since the early expression patterns of Poxm are almost identical in embryos of *D. melanogaster*, *D. erecta*, *D. pseudoobscura*, and *D. virilis* (Fig. 6), transcriptional regulation of *Poxm* during early embryonic stages may be conserved in these *Drosophila* species. Hence, by comparing the upstream regulatory sequences of *Poxm* among different *Drosophila* species, one may extract the sequences that are crucial for the formation of the early expression pattern of Poxm. Using sequence alignment of *um2*, I isolated *um2I* and *um2II*, which are highly conserved between *D. melanogaster* and *D. pseudoobscura* (Figs. 7 and 8). The functions of these newly isolated enhancers were tested *in vivo* by the use of transgenic flies containing *lacZ* reporters. With or without the natural linker sequence in between, *um2I_2II* and *um2I&2II* can drive *lacZ* expression in a way similar to *um2-lacZ* (Fig. 9). Moreover, as *um2δIδII-lacZ* does not show any specific expression pattern (Fig. 9D-F), the *um2* enhancer is not functional without *um2I* and *um2II*. These results indicate that *um2I* and *um2II* are both necessary and sufficient for early Poxm expression.

To examine whether *um2I* and *um2II* contain the Dpp-dependent silencer, the

lacZ reporters *um2I_2II-lacZ* and *um2I&2II-lacZ* were crossed into flies carrying *prd-Gal4* and *UAS-dpp*, which overexpress Dpp in every other segment. In both cases, expression of the *lacZ* reporters was strongly repressed by the overexpressed Dpp (Fig. 10). Therefore, *um2I&2II* contains not only activator binding sites but also the Dpp-dependent silencer.

Dpp overexpression in ectoderm does not lead to a complete dorsalization of the mesoderm

The repression of *um2I&2II-lacZ* in embryos expressing *prd-Gal4/UAS-dpp* may be caused by the effect that the mesoderm is completely dorsalized when Dpp is overexpressed in the ectoderm. To test this possibility, I analyzed the expression of the mesoderm marker Twi. When Dpp is overexpressed in every other segment of the ectoderm, Twi expression is weakened in these segments (Fig. 11A, D). In addition, when Dpp is overexpressed throughout the mesoderm under the control of *twi-Gal4*, the Twi expression in the abdominal segments is weaker than in wild-type embryos, but it is not completely abolished (Fig. 11B, D). This phenotype can be fully rescued by overexpressing DAD at the same time (Fig. 11C). These results suggest that *twi* can be partially repressed by Dpp. This repression is Dpp-specific and may involve components of the canonical Dpp signaling pathway. Because Twi is the crucial factor for the development of the somatic mesoderm (Baylies and Bate, 1996) where Poxm is expressed, the effect of the weakened Twi expression in embryos expressing *prd-Gal4/UAS-dpp* was analyzed after staining these embryos with anti-MHC. The muscle patterns are quite abnormal, especially the ventral and lateral muscles, but the somatic mesoderm does not disappear altogether (Fig. 12). These results imply that overexpression of Dpp in the ectoderm does cause the maldevelopment of the mesoderm, but does not lead to a complete dorsalization of the mesoderm.

Discussion

TKV, MAD, and DAD are all components of the canonical Dpp signaling pathway, their involvement in the repression of *Poxm* by Dpp indicates that this process may not be distinct from other reported cases. The most studied gene repressed by Dpp directly is *brinker* (*brk*). A minimal Dpp-dependent silencer containing a single binding site for each of the two signal mediators, MAD and Med, has been identified

in the *cis*-regulatory region upstream of *brk* (Pyrowolakis et al., 2004). The precise sequence and spacing of the MAD and Med binding sites allow the recruitment of the co-repressor Shn to form a protein-DNA complex that can repress the expression of *brk* effectively. Although the recruitment of Shn requires the presence of a specific nucleotide at a certain position of the silencer, Shn does not bind the silencer element by itself in the absence of MAD and Med (Pyrowolakis et al., 2004). This mechanism was also used in the repression by Dpp signaling of other genes, and the silencer element identified was proposed to be a *cis*-regulatory signature for Dpp-dependent repression without the involvement of cell type-specific transcription coregulators (Pyrowolakis et al., 2004). Thus, in all the reported cases, Shn is indispensable for Dpp-dependent repression. However, in *shn* null mutant embryos, the expression of Poxm is not affected. This is different from the situation in *dpp* null and hypomorphic mutants, where the expression of Poxm extends to the dorsal mesoderm. Therefore, factors other than Shn must be required to mediate the repression of *Poxm* by Dpp signaling in wild-type embryos.

What are these factors? To answer this question, one is faced with another related question: is the repression of *Poxm* by Dpp signaling direct, indirect, or both? Although *shn* mutants exhibit many phenotypes also found in *dpp* mutants, *dpp* null mutant embryos are more severely affected (Arora et al., 1995; Grieder et al., 1995; Staehling-Hampton et al., 1995). Therefore, there are indeed genes that are regulated by Dpp signaling independent of Shn. In *dpp* mutant embryos, these genes are either activated or inactivated ectopically, depending on how Dpp regulates them. It is possible that *Poxm* is regulated by some of these genes and thus is regulated by Dpp indirectly. If this is the case, these genes should be expressed in the mesoderm, their activation or repression by Dpp should not depend on Shn, and they should repress or activate *Poxm*. Although no gene is shown to fulfill all these requirements, there is one candidate, the *Six4/5* family member *D-Six4*. Initially expressed throughout the mesoderm, *D-Six4* mRNA is then restricted to ventral and lateral mesoderm by stage 10. In addition, in embryos overexpressing Dpp ectopically in the mesoderm, *D-Six4* is repressed (Clark et al., 2006). Thus, it was proposed that Dpp signaling represses *D-Six4* to restrict it to the ventral and lateral mesoderm (Clark et al., 2006), although it is not clear whether this repression depends on Shn. In addition, the expression of Poxm is largely reduced in *D-Six4* mutants (Zhang and Noll, unpublished results), which makes it possible for *D-Six4* to mediate the repression of *Poxm* by Dpp.

On the other hand, *Poxm* can be repressed by Dpp signaling directly. In *um2I&2II*, a single binding site for each of MAD and Med was found (Zhang and Noll, unpublished data). This is consistent with the conclusion that MAD is involved in the repression of *Poxm* by Dpp. Comparing the sequence of these binding sites with those in the Dpp-dependent silencer 2 (*SE2*) of *gsb*, one can see that the MAD binding sites are identical and the Med binding sites contain a difference in a single nucleotide (Zhang and Noll, unpublished data; Pyrowolakis et al., 2004). However, this different nucleotide is crucial neither for the formation of the *SE*/MAD/Med complex nor for the further recruitment of Shn to this complex (Pyrowolakis et al., 2004). Therefore, it is likely that the one nucleotide difference does not inactivate the Med binding site in *um2I&2II*. Interestingly, the spacing between the MAD and Med binding sites was shown to be important for Shn recruitment. Although the spacing mutants in which one or two nucleotides were inserted or deleted between the MAD and Med sites were still able to form a MAD/Med complex, only the element maintaining the natural 5 bp spacing can recruit Shn (Pyrowolakis et al., 2004). The spacing of MAD and Med binding sites in *um2I&2II* is 6 bp. Therefore, it is possible that this element cannot recruit Shn effectively and may recruit other yet unknown co-repressors to mediate the repression by Dpp independent of Shn.

It will be exciting to find out which of these possibilities is true, further experiments may help us to approach this aim.

References

1. Affolter, M and Basler, K. (2007). The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nat. Rev. Genet.* 8, 663-674.
2. Affolter, M., Marty, T., Vigano, M.A. and Jazwinska, A. (2001). Nuclear interpretation of Dpp signaling in *Drosophila*. *EMBO J.* 20, 3298-3305.

3. Arora, K. and Nusslein-Volhard, C. (1992). Altered mitotic domains reveal fate map changes in *Drosophila* embryos mutant for zygotic dorsoventral patterning genes. *Development* 114, 1003-1024.

4. Arora, K., Dai, H., Kazuko, S.G., Jamal, J., O'Connor, M.B., Letsou, A. and Warrior, R. (1995). The *Drosophila schnurri* gene acts in the Dpp/TGF beta

signaling pathway and encodes a transcription factor homologous to the human MBP family. *Cell* 81, 781-790.

5. Baylies, M.K. and Bate, M. (1996). *twist*: a myogenic switch in *Drosophila*. *Science* 272, 1481-1484.

6. Chen, C.R., Kang, Y., Siegel, P.M., and Massague, J. (2002). E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to *c-myc* repression. Cell *110*, 19–32.

7. Chen, J. (2003). Analysis of the transcriptional regulation and developmental functions of *Poxm* in *Drosophila melanogaster.* Ph.D. Thesis, University of Zürich.

8. Clark, I.B.N., Boyd, J., Hamilton, G., Finnegan, D.J. and Jarman, A.P. (2006). *D*-six4 plays a key role in patterning cell identities deriving from the *Drosophila* mesoderm. *Dev. Biol.* 294, 220-231.

9. Duan, H., Zhang, C., Chen, J., Sink, H., Frei, E. and Noll, M. (2007). A key role of *Pox meso* in somatic myogenesis of *Drosophila*. *Development* 134, 3985-3997.

10. Grieder, N.C., Nellen, D., Burke, R., Basler, K. and Affolter, M. (1995). *schnurri* is required for *Drosophila* Dpp signaling and encodes a zinc finger protein similar to the mammalian transcription factor PRDII-BF1. *Cell* 81, 791-800.

11. Gutjahr, T., Patel, N., Li, X., Goodman, C., and Noll, M. (1993). Analysis of the gooseberry locus in Drosophila embryos: gooseberry determines the cuticular pattern and activates gooseberry neuro. *Development* 118, 21-31.

12. Inoue, H., Imamura, T., Ishidou, Y., Takase, M., Udagawa, Y., Oka, Y., Tsuneizumi, K., Tabata, T., Miyazono, K. and Kawabata, M. (1998). Interplay of signal mediators of decapentaplegic (Dpp): molecular characterization of mothers against dpp, Medea, and daughters against dpp. *Mol. Biol. Cell* 9, 2145-2156.

13. Irish, V.F. and Gelbart, W.M. (1987). The decapentaplegic gene is required for dorsal-ventral patterning of the *Drosophila* embryo. *Genes. Dev.* 1, 868-879.

14. Kang, Y., Chen, C.R., and Massague, J. (2003). A self-enabling bound to DNA: insights on DNA binding in TGF-beta signaling. *Cell 94*, 585-594.

15. Kiehart, D.P. and Feghali, R. (1986). Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol*. 103, 1517-1525.

16. Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of brinker transcription. *Nature Cell Biol*. 2, 745-749.

17. Massagué, J. (1998). TGF-beta signal transduction. *Annu. Rev. Biochem.* 67, 753-791.

18. Müller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* 113, 221-233.

19. Nellen, D., Affolter, M. and Basler, K. (1994). Receptor serine/threonine kinases implicated in the control of *Drosophila* body pattern by decapentaplegic. *Cell* 78, 225-237.

20. Nellen, D., Burke, R., Struhl, G., Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* 85, 357-368.

21. Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J.L., Attisano, L., Szidonya, J., Cassill, J.A., Massague, J. and Hoffmann, F.M. (1994). Identification of two bone morphogenetic protein type I receptors in *Drosophila* and evidence that Brk25D is a decapentaplegic receptor. *Cell* 78, 239-250.

22. Pyrowolakis, G., Hartmann, B., Müller, B., Basler, K. and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during *Drosophila* development. *Dev. Cell* 7, 229-240.

23. Raftery, L. and Sutherland, D. (1999). TGF-β family signal transduction in *Drosophila* development: from Mad to Smads. *Dev. Biol.* 210**,** 251-268.

24. Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* 59, 1189-1202.

25. Rubin, G.M. and Spradling, A.C. (1982). *Science* 218, 348-353.

26. Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H. and Gelbart, W.M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in *Drosophila melanogaster*. *Genetics* 139, 1347-1358.

27. Spencer, F.A., Hoffmann, F.M. and Gelbart, W.M. (1982). Decapentaplegic: a gene complex affecting morphogenesis in *Drosophila melanogaster*. *Cell* 28, 451-461.

28. Staehling-Hampton, K., Hoffmann, F.M., Baylies, M.K., Rushton, E. and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* 372, 783-786.

29. Staehling-Hampton, K., Laughon, A.S. and Hoffmann, F.M. (1995). A *Drosophila* protein related to the human zinc finger transcription factor PRDII/MBPI/HIV-EP1 is required for dpp signaling. *Development* 121, 3393-3403.

30. St. Johnston, R.D., Hoffmann, F.M., Blackman, R.K., Segal, D., Grimaila, R., Padgett, R.W., Irick, H.A. and Gelbart, W.M. (1990). Molecular organization of the decapentaplegic gene in *Drosophila melanogaster*. *Genes Dev.* 4, 1114-1127.

31. Tracey, W.D.Jr., Ning, X., Klingler, M., Kramer, S.G. and Gergen, J.P. (2000). Quantitative analysis of gene function in the *Drosophila* embryo. *Genetics* 154, 273-284.

32. Wharton, K.A., Ray, R.P. and Gelbart, W.M. (1993). An activity gradient of decapentaplegic is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* 117, 807-822.

33. Xiao, H., Hrdlicka, L.A. and Nambu, J.R. (1996). Alternate functions of the *single-minded* and *rhomboid* genes in development of the *Drosophila* ventral neuroectoderm. *Mech. Dev.* 58**,** 65-74.

34. Zhang, Y., Ungar, A., Fresquez, C. and Holgrem, R. (1994). Ectopic expression of either *Drosophila gooseberry-distal* or *proximal* gene causes alterations of cell fate in the epidermis and central nervous system. *Development* 120, 1151-1161.

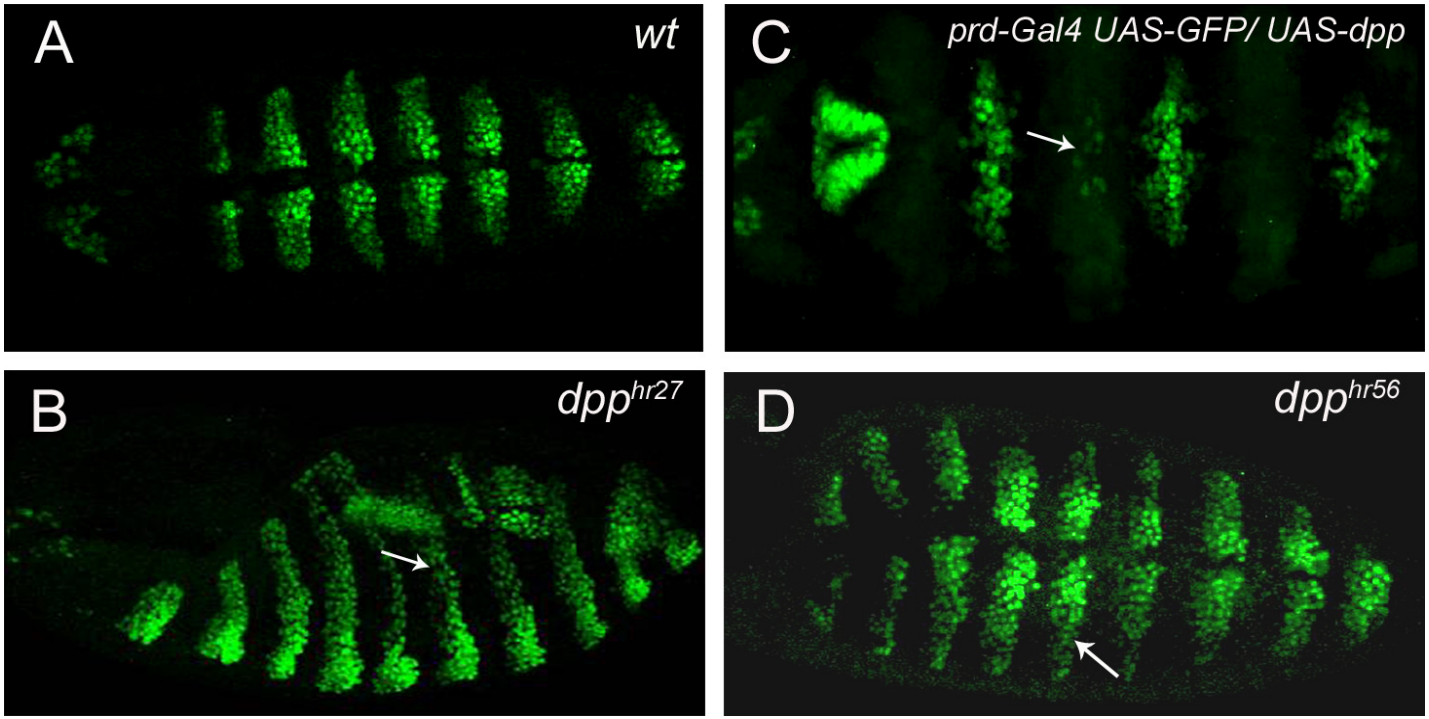


Fig. 1. Poxm expression is repressed by Dpp signaling. Wild-type (A), *dpp*^{hr27} (B), *prd-Gal4 UAS-GFP/UAS-dpp* (C), and *dpp*^{hr56} (D) embryos at stage 11 stained for Poxm protein are shown. Arrows indicate ectopic (B, D) or strongly reduced (C) Poxm expression caused by reduced levels of Dpp signaling (B, D) or ectopic Dpp expression (C). Ventral views (A, C, D) or a lateral view with dorsal side up (B) of embryos oriented with their anterior to the left are shown.

prd-Gal4 UAS-GFP/UAS-tkv^{QD} *twi-Gal4/+; UAS- tkv^{QD}/+*

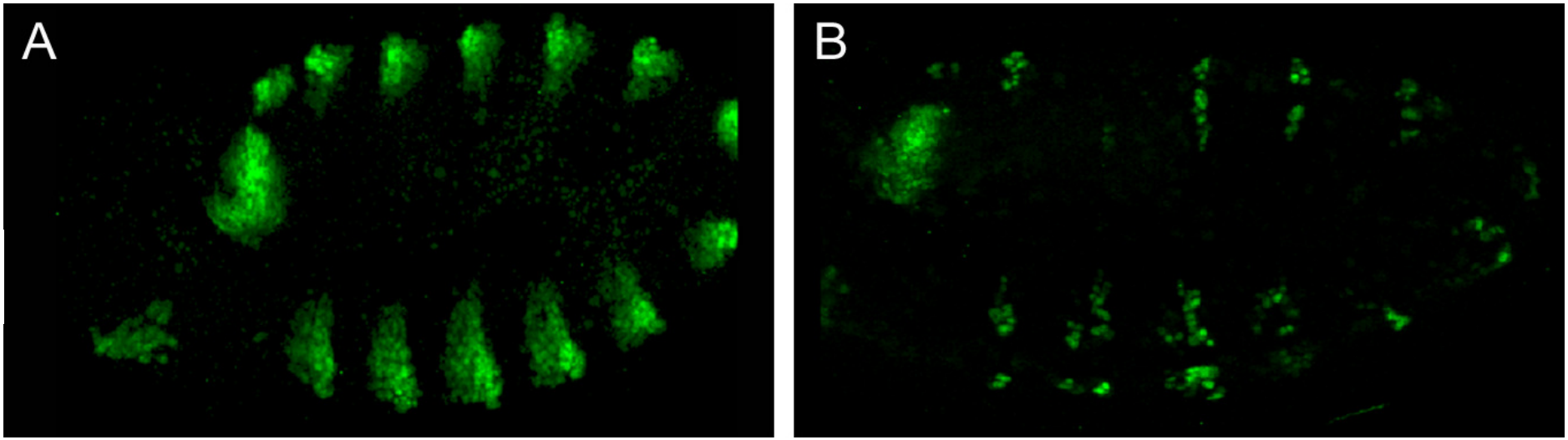


Fig. 2. Dpp signaling is required within the mesoderm to repress *Poxm*. *prd-Gal4 UAS-GFP/UAS-tkv^{QD}* (A), and *twi-Gal4/+; UAS- tkv^{QD}/+* (B) embryos at stage 11 stained for Poxm protein are shown. Embryos are oriented with their anterior to the left and dorsal side up.

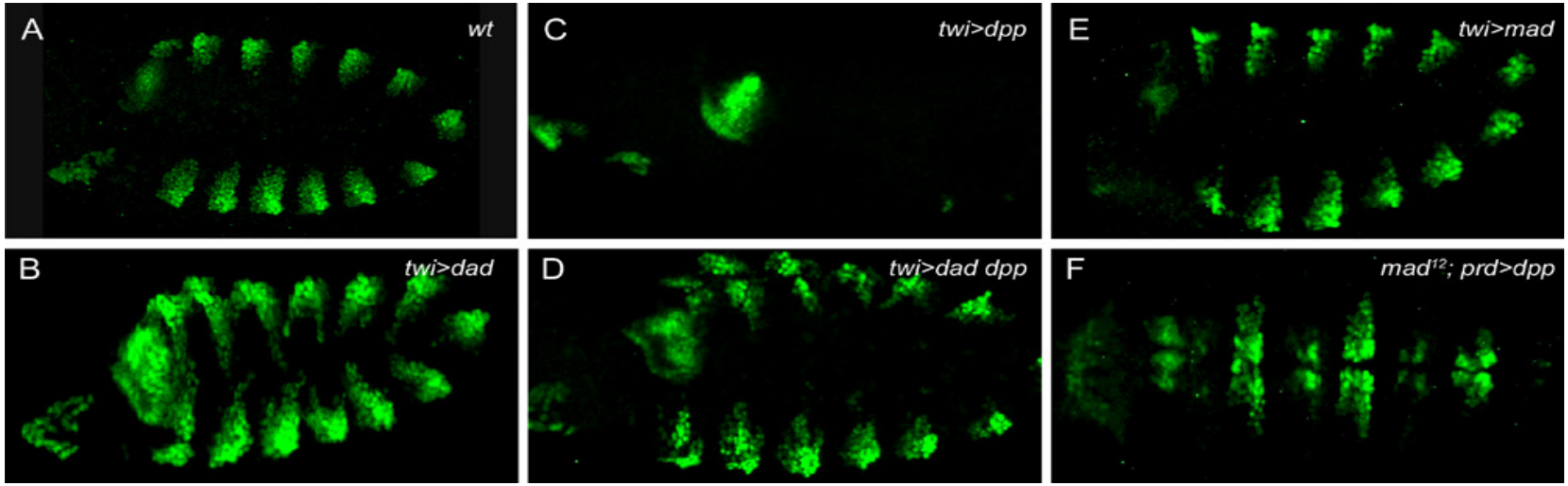


Fig. 3. Components of the canonical Dpp signaling pathway are involved in the repression of *Poxm*. Wild-type (A), *twi-Gal4/+*; *UAS-dad/+* (B), *twi-Gal4/+*; *UAS-dpp/+* (C), *twi-Gal4/+*; *UAS-dad/+*; *UAS-dpp/+* (D), *twi-Gal4/+*; *UAS-mad/+* (E), and mad^{12}; *prd-Gal4 UAS-GFP/UAS-dpp* (F) embryos at stage 11 and stained for Poxm protein are shown. Lateral views (A-E) or a ventral view (F) of embryos oriented with their anterior to the left are shown.

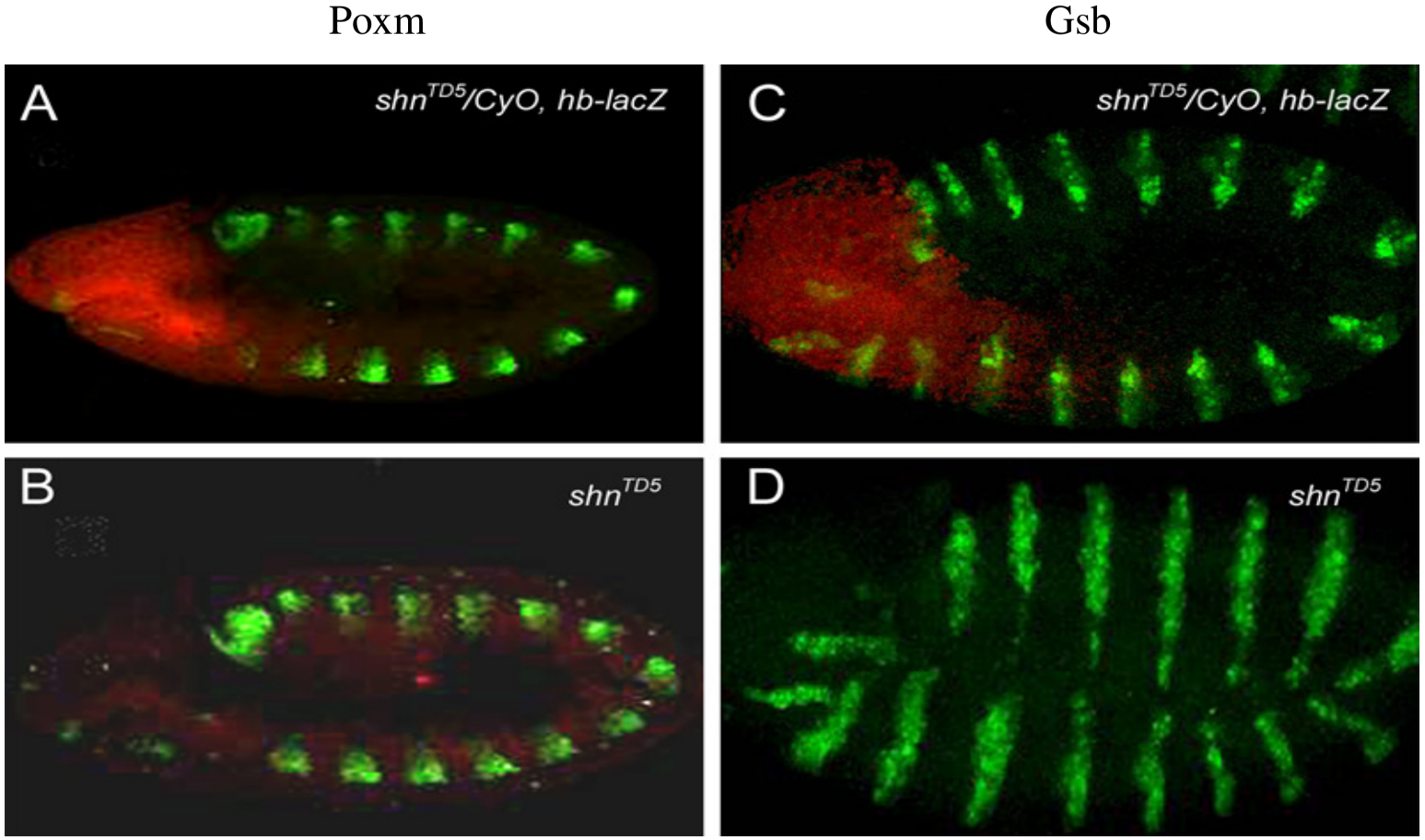


Fig. 4. Early Poxm expression is not affected in *shn* mutants. Lateral views of *shn*^{TD5}*/CyO, hb-lacZ* (A, C) and *shn*^{TD5} (B, D) embryos at stage 11, stained for Poxm (A, B) or Gsb (C, D) in green and β -Galactosidase (A-D) in red, are shown. All the embryos are oriented with their anterior to the left and dorsal side up.

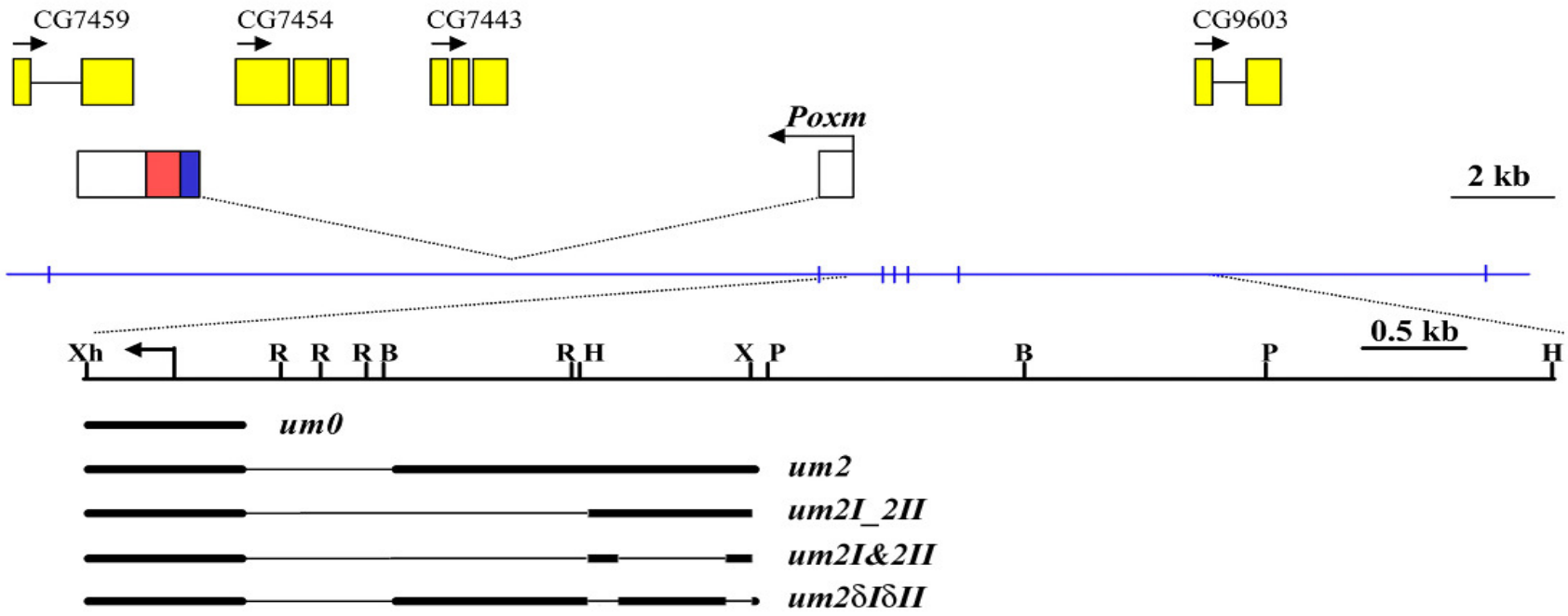


Fig. 5. Map of *Poxm* locus and *Poxm-lacZ* reporter constructs. The *Poxm* locus and neighboring tanscripts (yellow) are illustrated above an *Eco*RI map marked as blue line. At the *Poxm* locus, introns and exons are indicated with the paired domain marked in blue and the C-terminal domains in red. Below the *Eco*RI map, an enlarged restriction map of the 5' portion of the *Poxm* leader and the adjacent upsream region is shown (only selected restriction sites are indicated), below which different *cis*-regulatory regions used in various *Poxm-lacZ* reporter constructs are shown as black bars. B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; X, *Xba*I; Xh, *Xho*I.

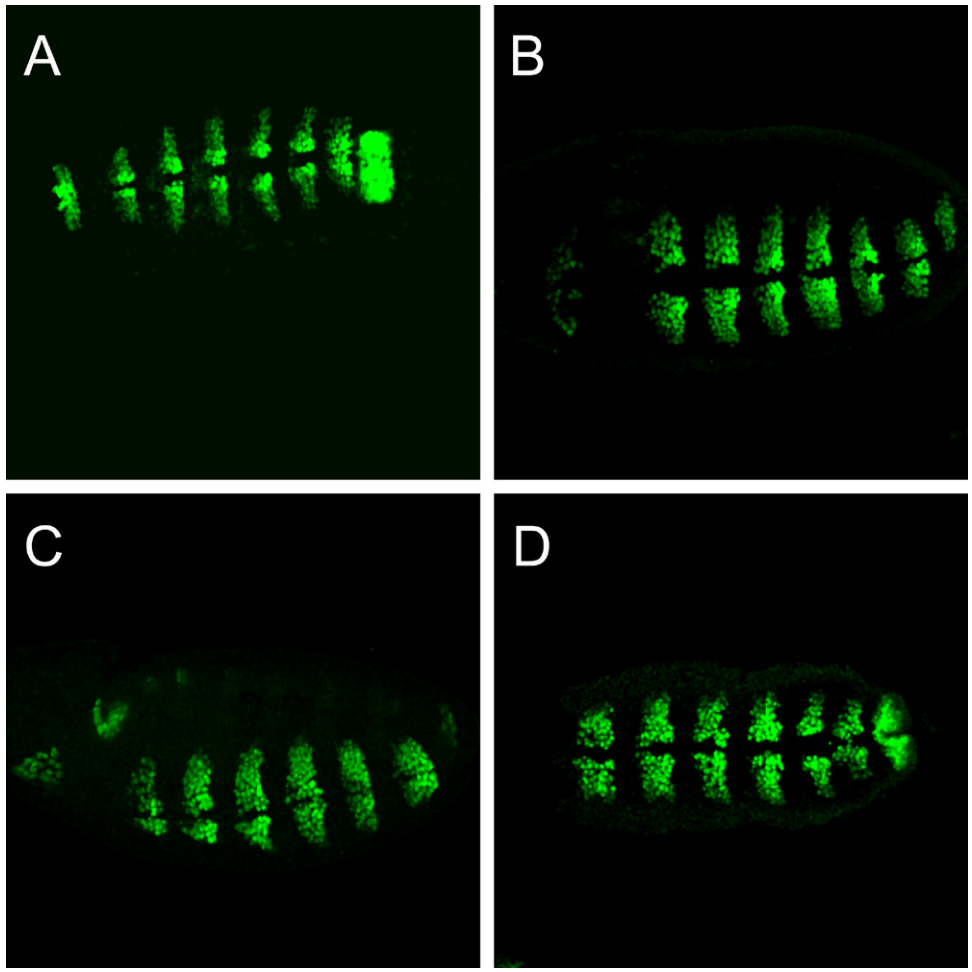


Fig. 6. Early Poxm expression pattern is conserved among different *Drosophila* species. *D. melanogaster* (A), *D. erecta* (B), *D. pseudoobscura* (C), and *D. virilis* (D) embryos at stage 11 stained for Poxm protein of *D. melanogaster* are shown. All embryos are oriented with their anterior to the left.

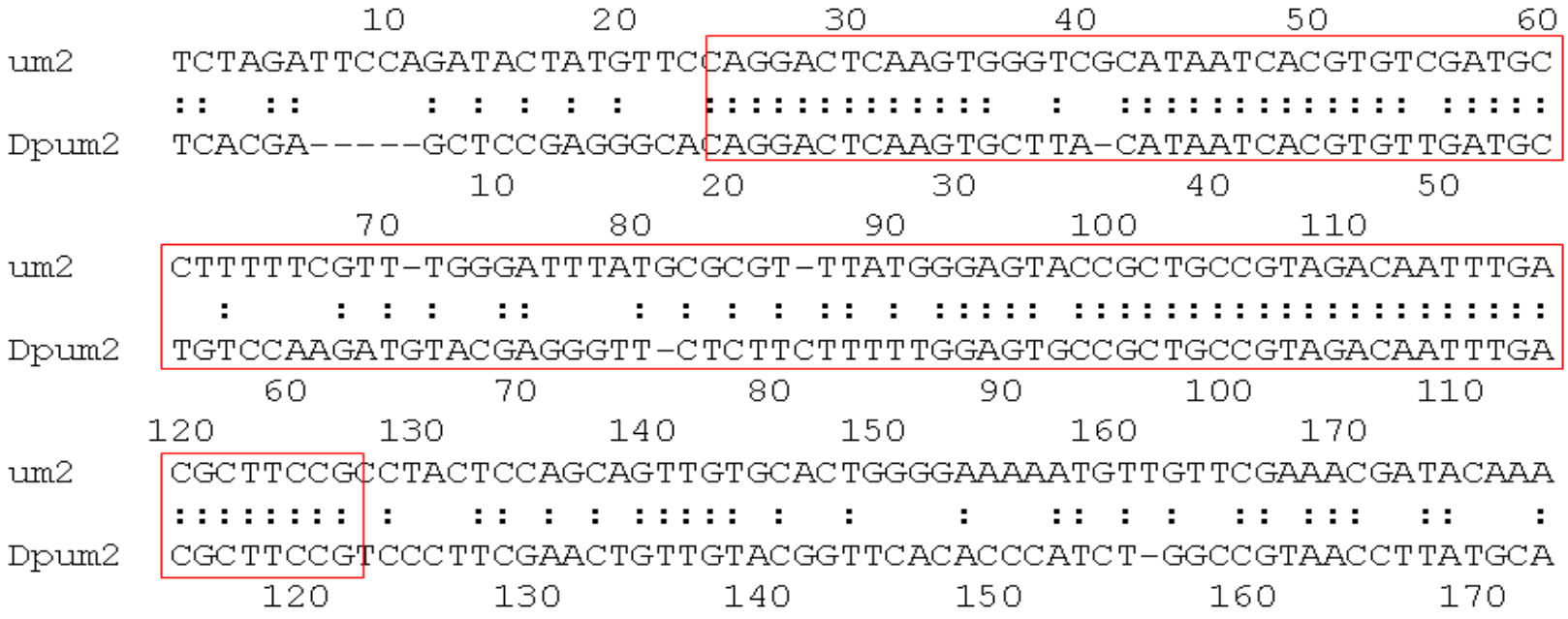


Fig. 7. Alignment of the *cis*-regulatory sequences from *D. melanogaster* (*um*2) and *D. pseudoobscura* (*Dpum*2). A highly conserved sequence (*um*2I) is boxed. Dashes indicate nucleotides missing in the *um*2 or *Dpum*2 sequence.

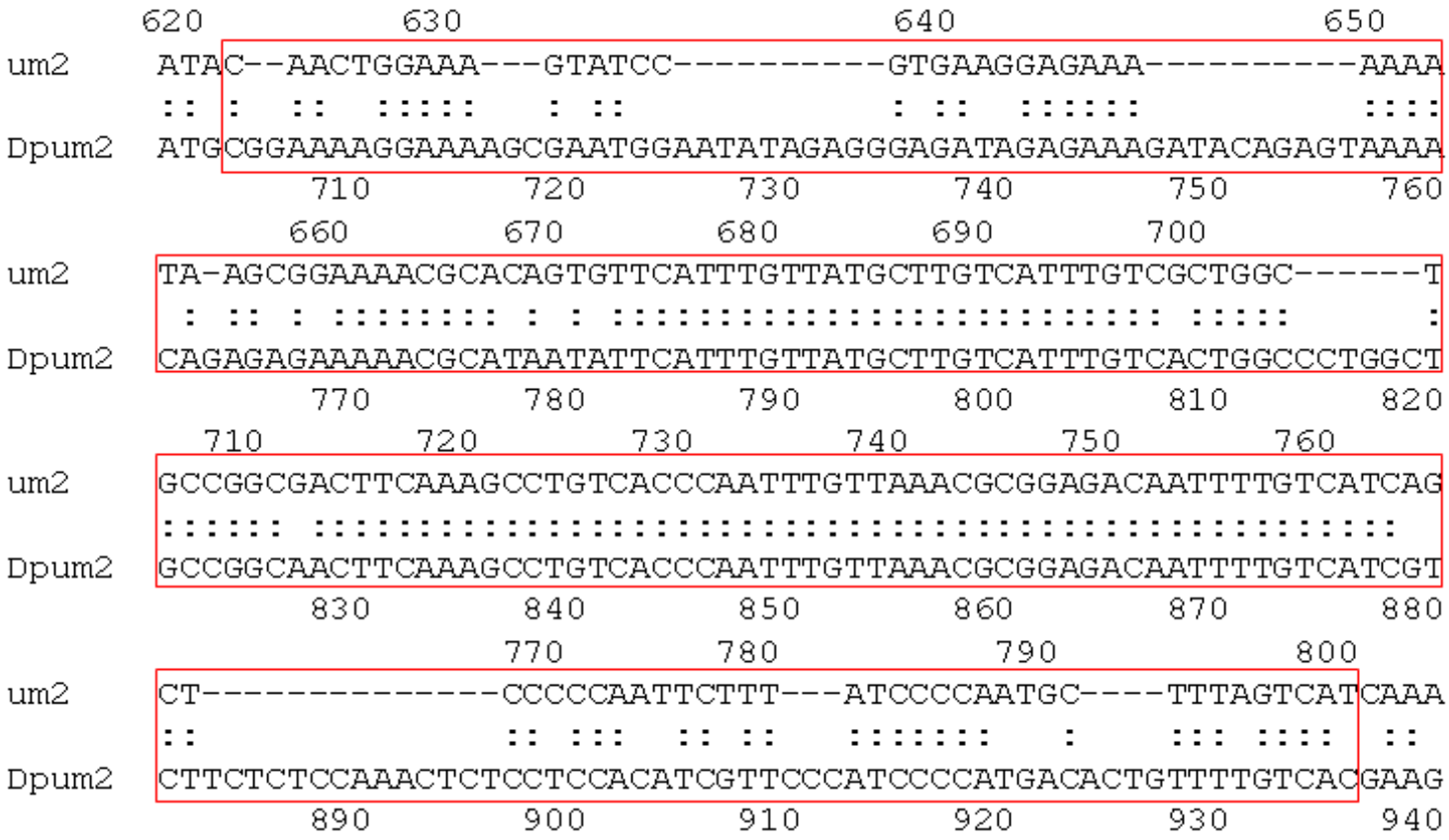


Fig. 8. Alignment of the *cis*-regulatory sequences from *D. melanogaster* (*um*2) and *D. pseudoobscura* (*Dpum*2). A highly conserved sequence (*um*2II) is boxed. Dashes indicate nucleotides missing in the *um*2 or *Dpum*2 sequence.

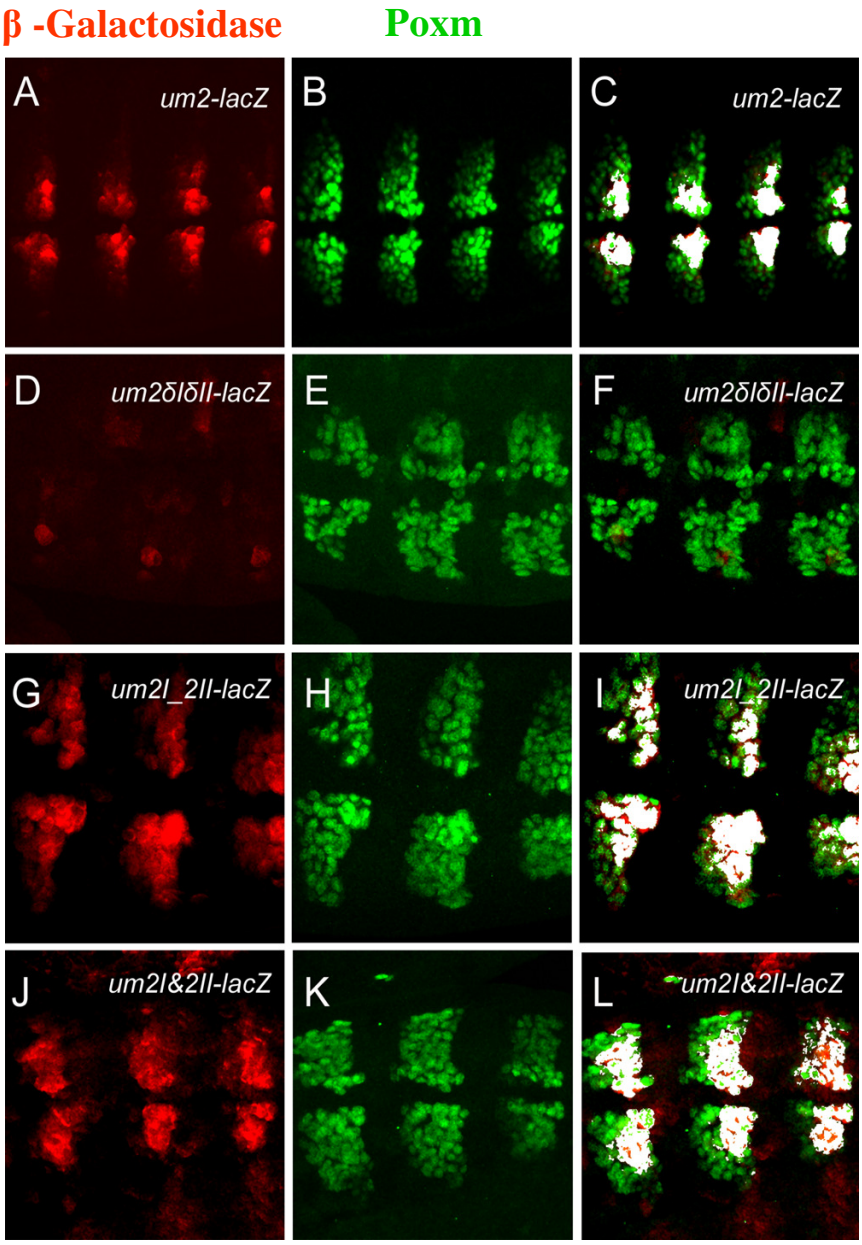


Fig. 9. Coexpression of *Poxm* and *Poxm-lacZ* reporter genes in the somatic mesoderm. Expression of β -Galactosidase (A, D, G, J) and Poxm protein (B, E, H, K) in late stage 10 (A-C) or stage 11 (D-L) embryos is visualized by antibody staining. Cells in which Poxm and β -Galactosidase colocalize are highlighted in white by the use of the image processing program ImageJ (C, F, I, L). All the segments are oriented with their anterior to the left. The *Poxm-lacZ* reporter constructs used (indicated in the left panel for each row of panels) are shown in Fig. 5

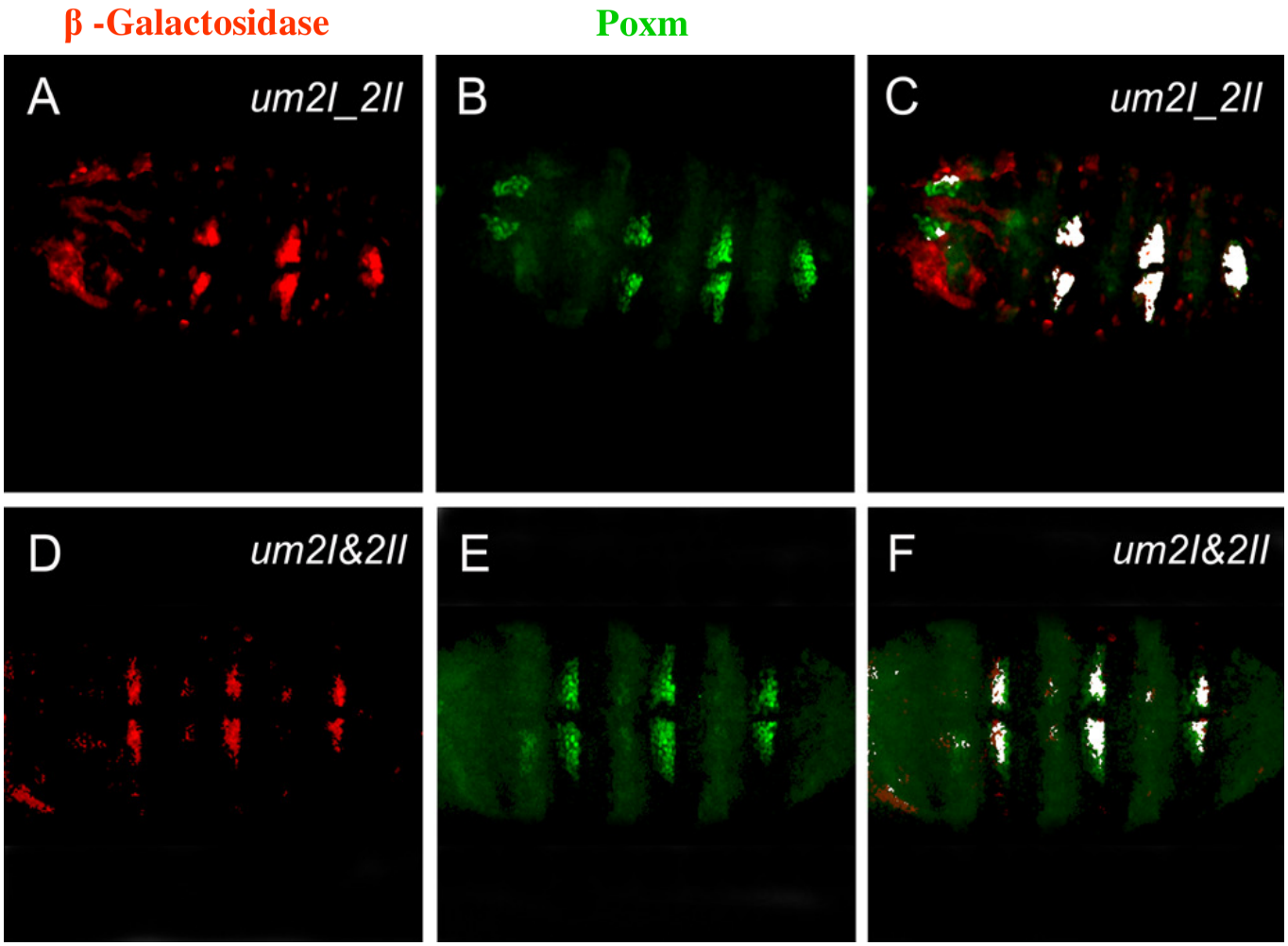


Fig.10. β -Galactosidase expression under the control of the early *Poxm* enhancer is regulated like endogenous *Poxm* by Dpp signaling. Expression of β -galactosidase (A, D) and Poxm protein (B, E) in stage 11 embryos is visualized by antibody staining. Cells that co-express Poxm and β -Galactosidase are highlighted in white by the use of the image processing program ImageJ (C, F). All embryos are oriented with their anterior to the left.

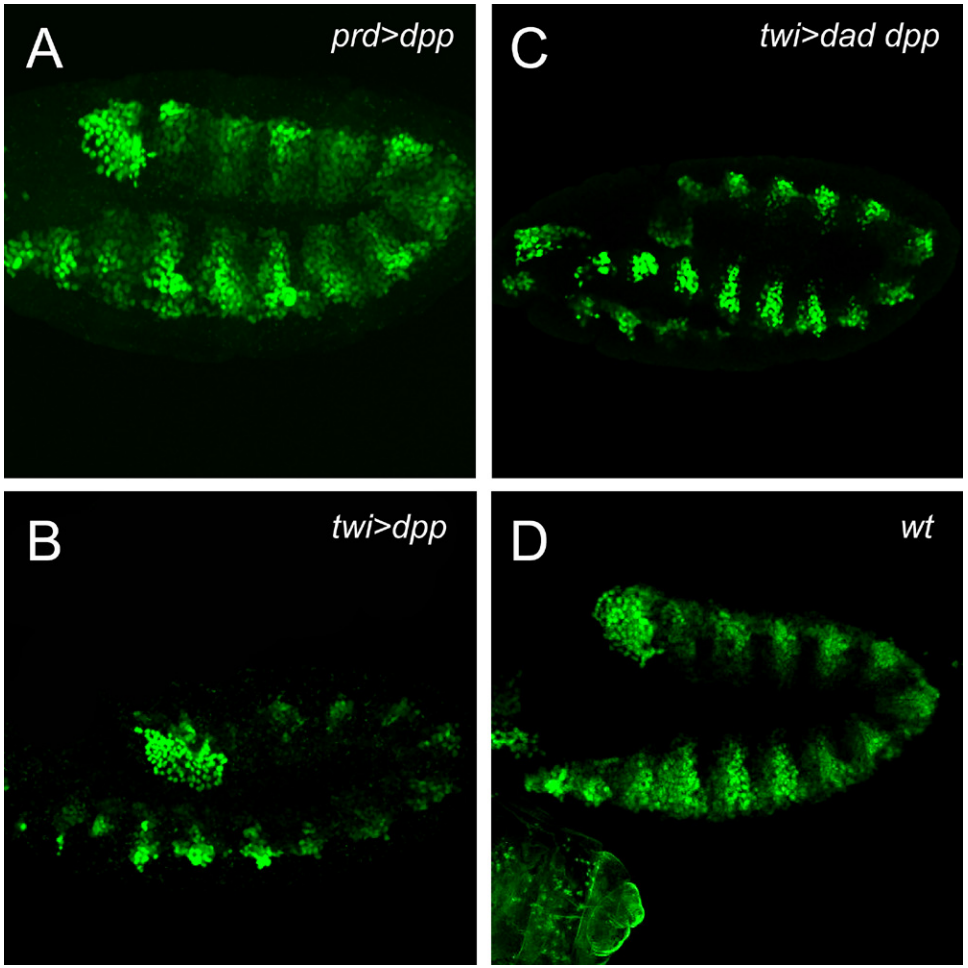


Fig.11. Twist expression can be repressed by ectopic Dpp signaling. *prd-Gal4 UAS-GFP/UAS-dpp* (A), *twi-Gal4/+*; *UAS-dpp/+* (B), *twi-Gal4/+*; *UAS-dad/+*; *UAS-dpp/+* (C), and wild-type (D) embryos at stage 11 and stained for Twist are shown. All embryos are oriented with their anterior to the left and dorsal side up.

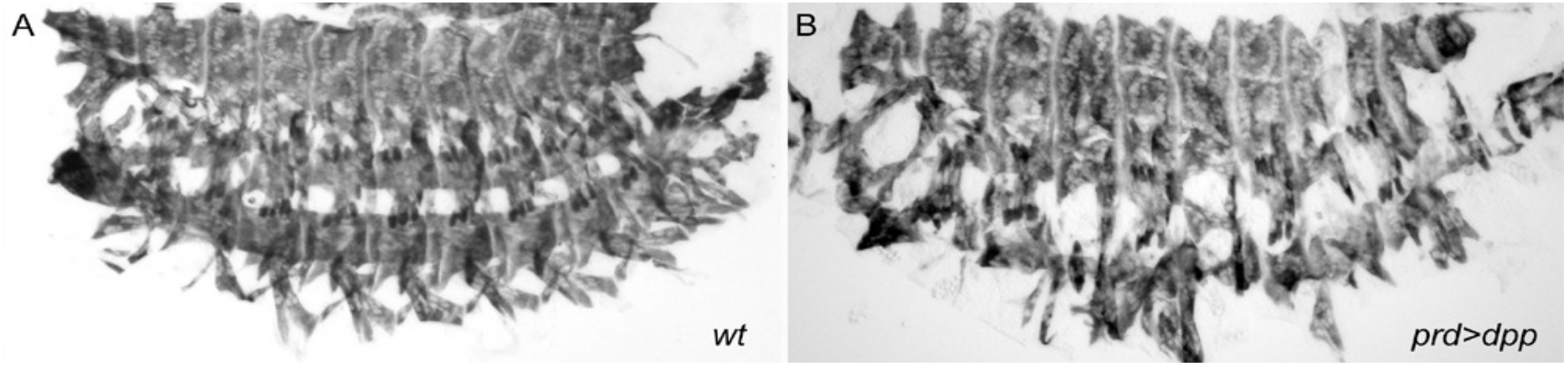


Fig.12. The pattern of somatic body wall muscles is affected but not abolished by ectopic Dpp signaling. Muscle phenotypes resulting from ectopic Dpp signaling were visualized by the use of an anti-MHC antiserum in stage 16 wild-type (A) and *prd-Gal4 UAS-GFP/UAS-dpp* (B) embryos. The embryos are oriented with their anterior to the left and dorsal side up.

Chapter 5

Conclusions

I began my studies trying to understand why certain genes are expressed in particular tissues during specific developmental stages. In other words, I aksed myself: what functions do these genes serve in tissue development and how are their expression patterns regulated by upstream regulators? Muscle patterning of *Drosophila* embryos is an ideal system to study these problems: on the one hand, many genes and signaling pathways involved in this process are known from studies over the years; on the other hand, questions concerning the functions and regulations of key players in this process remain to be answered. In this thesis, I analyzed the functions of the Pax genes *Poxm* and *gsb* during *Drosophila* embryonic myogenesis and studied their regulatory and functional relationships by placing them in the broader context of their gene network. In addition, I tried to connect the actions of external signaling pathways with the regulation of trancription factors by addressing the long-standing question through which mechanism *Poxm* is repressed by Dpp signaling.

The *Pax1/9* homolog *Poxm* plays a key role in somatic myogenesis of *Drosophila*

A time study of Poxm expression patterns shows that it is expressed in the ventral and lateral regions of the somatic mesodem during early embryonic stages and in a few muscle founder cells during later stages. These results suggest that *Poxm* has distinct functions during different stages of myogenesis. Indeed, while most of the ventral and lateral muscles affected in *Poxm* null mutants are rescued by a transgene expressing *Poxm* only during the early myogenic stages, the muscles expressing Poxm in their founders during late myogenic stages still appear abnormal. Thus, *Poxm* plays a dual role during *Drosophila* embryonic myogenesis: it exerts an early function to specify within the high Twi domain a subdomain of competence for lateral and ventral muscle development and a late function important for founder specification and

muscle differentiation that is characteristic for muscle identity genes. The early function of *Poxm* is partially redundant with the functions of *l(1)sc*. Together, they are part of the gene network controling specification of cell fates during early myogenic stages.

The *Pax3/7* homolog *gsb* is a new player in the *Pax-Six* gene network of *Drosophila* embryonic myogenesis

Like its vertebrate homologs *Pax3* and *Pax7*, *gsb* is also involved in myogenesis. It is expressed in a lateral region of the somatic mesoderm from stage 10 to 12. However, unlike *Poxm*, it is not expressed in differentiated muscle fibers during late embryonic stages. Loss-of-function mutants of *gsb* exhibit severe muscle phenotypes: most of the ventral muscles are either lost or abnormal. Thus, *gsb* is another Pax gene involved in the *Pax-Six* gene network of *Drosophila* embryonic myogenesis. While the mesodermal Gsb expression is affected in neither *Poxm* nor *D-Six4* mutants, Gsb acts as an activator of *Poxm*, since the expression of Poxm is down-regulated in *gsb* mutants. When Gsb is overexpressed throughout the mesoderm, the early expression of Poxm is not affected and all muslces but a few lateral ones develop normally. Thus, although *gsb* is necessary for myogenesis in wild-type embryos, it is not sufficient to cause maldevelopment of most muscles when ectopically overexpressed in the mesoderm.

Dpp signaling represses *Poxm* through a Shn-independent mechanism by acting on a *cis*-regulatory element of *Poxm*

Poxm expands to the dorsal mesoderm in both *dpp* null and hypomorphic mutants. In addition, the expression of Poxm is suppressed in embryos overexpressing Dpp ectopically. Therefore, Dpp signaling not only functions to restrict Poxm expression to the lateral and ventral regions of the mesoderm, but can also repress the expression of Poxm when ectopically activated ventrally. This activation of the Dpp signaling pathway has to happen in cells within the mesoderm because when Dpp signaling was only ectopically activated in ectodermal cells by the overexpression of the constitutively active Dpp receptor TKV^{QD}, Poxm expression was not affected. Interestingly, although components of the canonical Dpp signaling pathway, MAD and TKV, are involved in the repression of Poxm by Dpp, this process is independent of the co-repressor Shn. Therefore, it is possible that Poxm is repressed by Dpp

signaling by a yet unknown mechanism employing a co-regulator different from Shn. The mechanism functions by acting on a short Dpp dependent *cis*-regulatory element of *Poxm*. Putative binding sites for both MAD and Med were identified in this element, raising the possibility that *Poxm* is repressed by Dpp signaling directly.

Curriculum Vitae

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Publications:

1. Duan, H.*, **Zhang, C.***, Chen, J., Sink, H., Frei, E., and Noll, M. (2007). **A key role of *Pox meso* in somatic myogenesis of *Drosophila.*** *Development* 134: 3985-3997.(* These authors contributed equally to this work.)

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